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~~Molecular Aspects of Bid and Mcl-1 in Apoptosis~~

STUDIES ON THE ROLE OF BID AND MCL-1 IN THE REGULATION OF APOPTOSIS.

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Abstract

The Bcl-2 family of proteins are critical regulators of mitochondrial function and deregulation during apoptosis. To this effect, the 'BH3-only' members of this family play an important role in initiating many of the apoptotic signalling pathways. This thesis focuses on the BH3-only protein Bid. We have used the active form of Bid, tBid, as bait in a yeast two-hybrid assay to identify novel interaction partners for this protein. In combination with a GST pull-down assay we identified two novel interaction partners for tBid. The proteins identified were Mcl-1 and EID-1.

Mcl-1 is an anti-apoptotic Bcl-2 family member, although its role in apoptosis has not been as extensively studied as other anti-apoptotic proteins such as Bcl-2 and Bcl-X_L.

EID-1 was first identified as a pRb interacting protein that has been shown to inhibit differentiation of myoblasts. However, there is no data to suggest that EID-1 may have a role in apoptosis, and to date all data suggests that the protein has a nuclear function rather than a cytoplasmic one.

During the course of this work, we observed that Mcl-1 is cleaved during apoptosis of Jurkat T cells. We have characterised this cleavage and found it to be as a result of caspase cleavage. There are two caspase cleavage sites in Mcl-1. These occur at Asp^{127} and Asp^{157}. Many Bcl-2 family members are cleaved by caspases during apoptosis. In particular, anti-apoptotic family members cleaved by caspases are converted into pro-apoptotic proteins. In contrast to this we did not observe any pro-apoptotic activity associated with the cleavage fragments. We also found that the PEST region in Mcl-1 did not regulate its half-life in FDCP-1 cells, confirming results already observed in U937 cells.

While further work needs to be carried out, the results presented here provide a number of important observations that may help to further the understanding of how both Bid and Mcl-1 contribute to apoptosis.

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I would like to express my gratitude to Dr. Hugh Brady, my supervisor, for his help and encouragement over the past number of years.

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Finally, I wish to thanks my family for all their support, encouragement and understanding, I am forever indebted to them.

This thesis is dedicated to my parents

Mam and Dad

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ABBREVIATIONS

3-AT	3-amino-1,2,4-triazole
AICD	Activation-induced cell death
AIF	Apoptosis inducing factor
AP-l	Activator Protein 1
Apaf-1	Apoptotic protease activating factor-1
Bad	Bcl-2/Bcl-XL associating death effector
BH	Bcl-2 homology
Bid	*B*H3 *i*nteracting domain *d*eath agonist
Bim	*B*cl-2 *i*nteracting *m*ediator of cell death
BIR	Baculoviral IAP repeat
BSA	Bovine serum albumin
$CaC1_2$	Calcium chloride
CARD	Caspase activation and recruitment domain
CBB	Coomassie brilliant blue
cDNA	Complementary DNA
cm	Centimeter
CMML	Chronic myelomonocytic leukaemia
CO_2	Carbon dioxide
CREB	Cyclin response element binding
CTL	Cytotoxic T lymphocyte
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DD	Death domain
DISC	Death induced signalling complex
DR	Death receptor

DTT	Dithiothreitol
E.coli	Escherichia coli
ECL	Enhanced chemiluminescence
EID-1	E1A-like inhibitor of differentiation 1
GFP	Green fluorescent protein
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
FACS	Fluorescence activated cell sorter
FADD	Fas-associated death domain
FasL	Fas ligand
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FLIP	FADD-like interleukin-1β-converting enzyme
HA	Hemagglutinin
HRP	Horseradish peroxidase
IAP	Inhibitor of apoptosis
IgG	Immunoglobulin G
IL	Interleukin
IP	Immunoprecipitation
IPTG	Isopropyl-beta-D-thiogalactosidase
JNK	Jun N-terminal kinase
K_2PO_4	Potassium phosphate
kb	Kilobases
KCl	Potassium chloride
kDa	Kilodalton
KH_2PO_4	Potassium hydrogen phosphate
KOH	Potassium hydroxide
LB-agar	Luria-Bertini agar
L-broth	Luria broth
M	Molar
Mcl-1	Myeloid cell leukaemia 1
MEF	Mouse embryo fibroblast
mg	Milligrams

$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
ml	Millilitres
mM	Millimolar
mRNA	Messenger RNA
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NF-κB	Nuclear regulator factor-kappa B
ng	Nanograms
NGF	Nerve growth factor
nM	Nanomolar
Noxa	
NP40	Nonidet P 40
O.D.	Optical density
°C	Degrees celsius
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PE	Phycoerythrin
pH	Pleckstrin homology
PI 3-kinase	Phosphatidyl inositol 3-kinase
PI	Propidium iodide
PIG	p53 induced gene
PMA	Phorbol12-myristic l3-acetate
PUMA	
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SMAC	Second mitochondrial activator of caspases
SODD	Silencer of death domain
TAE	Tris acetate EDTA
tBid	Truncated Bid

TE	Tris EDTA
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRADD	TNFR-l-associated death domain
TRAF	TNF receptor associated factor
u.v.	Ultraviolet
V	Volts
VDAC	Voltage dependent anion channel
v/v	Volume per volume
w/v	Weight per volume
WB	Western blot
WCL	Whole cell lysate
YNB	Yeast nitrogen base
µg	Micrograms
µl	Microliters
µM	Micromolar

1 - Introduction

Tissue homeostasis requires that many aspects of cell growth and proliferation be carefully controlled. Normal growth is controlled at a number of different levels within cells and when problems arise they can be dealt with in a number of different ways. One way in which this control is exerted is through programmed cell death or apoptosis. This is a genetically controlled cell suicide that has proven to be very complex in terms of both its regulation and execution. Regulation of tissue development in this manner has proven to be vital for normal development and tissue homeostasis as well as being an important response to various cellular stresses.

1.1 – Apoptosis

Apoptosis as we know it today was first observed by Kerr, Wylie and Currie in 1972 (Kerr et al., 1972). The process has very distinctive morphological changes associated with it. Cells initially condense and the cytoplasm becomes quite concentrated and thick, the nuclear membrane breaks down and the cell's DNA condenses and becomes fragmented, with the cell membrane blebbing into apoptotic bodies.

These macroscopic features of apoptosis are controlled very carefully at a molecular level. Cells constantly receive signals that promote cell survival and growth. As well as signals that promote apoptosis directly, removal or withdrawal of such survival signals can initiate apoptosis. Death receptors expressed on the cell surface transduce signals from outside the cell and administer a direct signal to initiate apoptosis (Locksley et al., 2001), while checkpoint proteins such as p53 can control and initiate signals frxom internal stresses by upregulating pro-apoptotic genes that initiate apoptosis (Fridman and Lowe, 2003).

The result of these initiation signals is ultimately the activation of a family of proteases known as caspases.

1.1.1 – Caspases

Caspases (*c*ysteinyl *asp*artate-specific prote*ases*) are the main workhorses of apoptosis (Earnshaw et al., 1999). Once activated they carry out much of the work required for successful completion of the apoptotic program. As well as having the ability to cleave and activate other caspases, they cleave and activate other molecules within the cell. This converts neutral or anti-apoptotic proteins into strong pro-apoptotic proteins with specific roles in apoptosis (Nicholson, 1999).

There is now a large number of mammalian caspases identified and these can be placed into two main groups – initiator caspases or effector caspases. Initiator caspases are upstream caspases and initiate the caspase cascade (Earnshaw et al., 1999). This group includes caspase-8, -2 and –10, which are the first caspases activated in the extrinsic pathway at the DISC (Peter and Krammer, 2003). Caspase-9 is also an initiator caspase, being the initial caspase activated after mitochondrial deregulation (Adrain and Martin, 2001). The effector caspases, also known as executioner caspases, are downstream caspases that cleave a very broad range of cellular substrates (Earnshaw et al., 1999). Caspases in this group include caspase-3, -6, and -7.

All caspases cleave after an aspartic acid residue (termed P1), with the amino acids at positions P2, P3 and P4 preceding P1 at the cleavage site also being important in determining what constitutes a cleavage site (Rano et al., 1997; Thornberry et al., 1997). Caspase-3 and -7 cleavage sites generally have the consensus sequence DXXD. Although this is the most common caspase cleavage motif there are many caspase-3 and -7 cleavage sites that do not share this motif (Nicholson, 1999). Consensus cleavage sites for other caspases can differ from that of caspase-3 although they all share the requirement for an aspartic acid residue at position P1 and often a number of different caspases can cleave at the same site.

Regulation of caspases and caspase activity is critical to normal cellular homeostasis. To leave such proteases unchecked can have dire consequences for the cell.

1.2 – Apoptosis in Development and Disease

Apoptosis plays an important role in development of all multicellular organisms. It is a highly conserved process that is shared by simple organisms such as the nematode worm *Caenorhabditis elegans*, and the more complex fruit-fly *Drosophila melanogaster* as well as vertebrates (Fig. 1-1). During normal development of *C. elegans* 1090 cells are generated. The same 131 of these cells die in a specific and reproducible manner to generate the mature worm. Studies of death in these cells have identified a simple apoptotic pathway that appears to be a basic, primitive model of what happens in higher organisms. Apoptosis in *Drosophila* is a more complex event and includes a number of genes (*rpr*, *grim*, and *hid*) that have little homology to apoptotic genes identified in either the worm or mammals. Interestingly apoptosis in both *C. elegans* and *Drosophila* appears to be limited to early life and does not appear to have a role beyond development of the mature organism. In contrast, the process of programmed cell death in mammals is not only important during embryonic and foetal development but it has a functional importance for many biological processes throughout the lifetime of the mammal. Apoptosis is a vital tool for proper homeostasis and maintenance of biological systems such as the nervous and immune systems (Becker and Bonni, 2004; Opferman and Korsmeyer, 2003; Zahir and Weaver, 2004).

Throughout an organism's life span its cells accumulate damage to their genome. This may be as a result of exposure to various genotoxic agents as well as the normal process of ageing. Over time, such damage may lead to impairment of control mechanisms within cells. Disruption in the balance of molecules that control apoptosis can have adverse consequences for the cell and contribute to the pathogenesis of a number of disease types. Such changes that lead to the promotion of cell survival can be a major stepping stone to the development of cancer (Hanahan and Weinberg, 2000), while changes that promote apoptosis can contribute to the pathogenesis of many neurodegenerative diseases (Mattson, 2000).

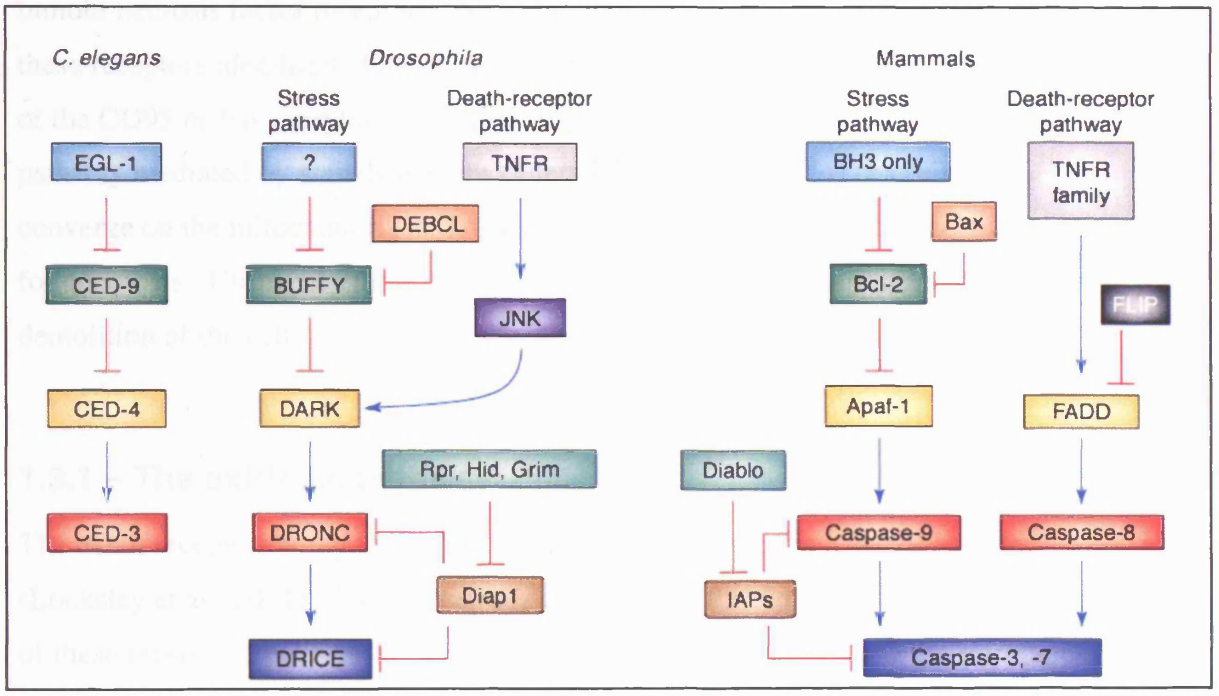


Figure 1-1: Conserved components of the cell-death machinery. The diagram above shows conserved pathways in *C. elegans*, *Drosophila* and mammals that are key to caspase activation. Analogous colours represent homologus proteins between species. The pathway in *C. elegans* is a much simpler version of the pathway found in both *Drosophila* and mammals.

1.3 – Apoptotic pathways

Execution of apoptosis is signalled through two main pathways. One is an extrinsic pathway, whereby external signals are transduced across the cell membrane by a family of receptors known as death receptors. These death receptors belong to the tumour necrosis factor receptor (TNFR) family. There are now a large number of these receptors identified. One of best-characterised death receptor pathways is that of the CD95 or Fas receptor. The other main apoptotic pathway is an intrinsic pathway mediated by signals that are generated within the cell. These signals converge on the mitochondria and result in release of mitochondrial proteins required for apoptosis. Ultimately these pathways result in caspase activation and subsequent demolition of the cell.

1.3.1 – The extrinsic pathway

The death receptor family, or TNFR family, now consists of at least 29 receptors (Locksley et al., 2001). These receptors are found on the surface of cells with some of these receptors being tissue specific (e.g. the neuronal specific p75 neurotrophic receptor) and others more ubiquitous (e.g. the Fas receptor). All death receptors have the same basic structure (Locksley et al., 2001). They contain a cysteine-rich extracellular domain, and usually have a death domain (DD) in their cytoplasmic regions. Generally there is also good homology between cytoplasmic domains of these receptors. Ligation of a death ligand to its corresponding death receptor results in oligomerisation of the receptor. Internally this results in the formation of a death induced signalling complex (DISC) (Peter and Krammer, 2003). This DISC allows for the activation of initiator caspases that then go on to activate downstream executioner caspases (Locksley et al., 2001; Peter and Krammer, 2003).

The Fas (CD95) receptor has been one of the best studied death receptors to date and is a good example of how these receptors signal apoptosis (Krammer, 2000; Nagata, 1999). This pathway is outlined in Figure 1-2. Binding of Fas ligand (FasL) to the receptor results in trimerisation of the receptor. This brings the Fas receptor DDs into close contact with each other and acts as a platform for the assembly of the DISC

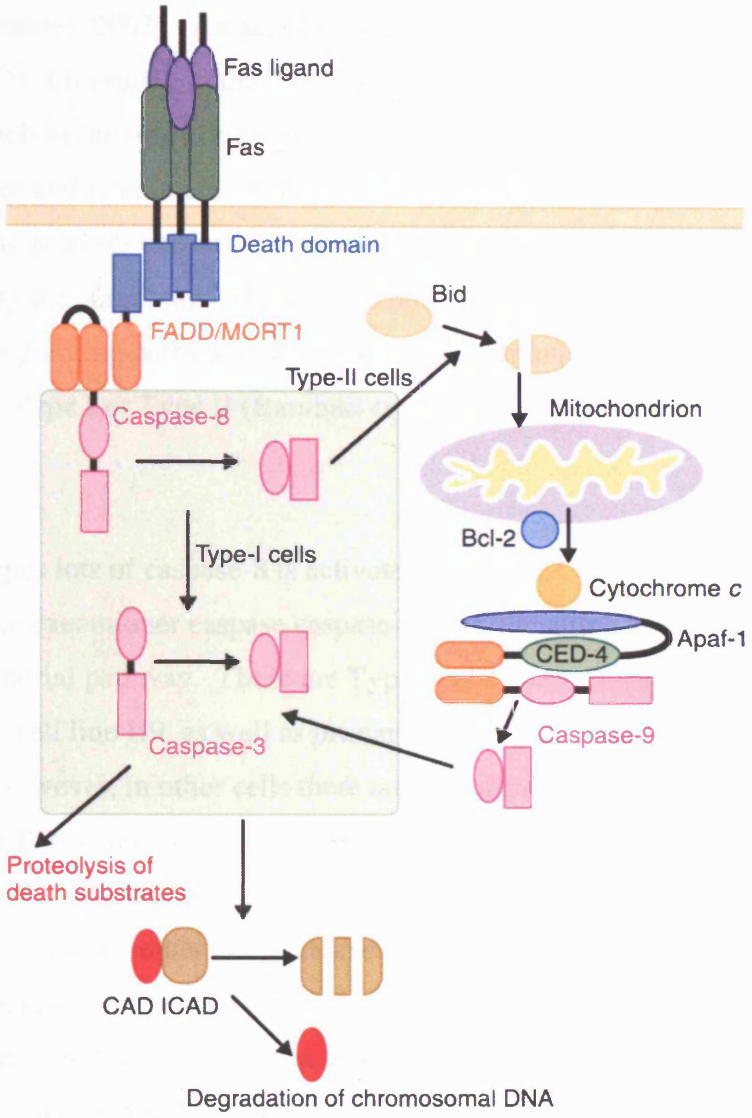


Figure 1-2: Extrinsic signalling via the CD95 (Fas) receptor pathway. This figure outlines the events that occur during Fas mediated apoptosis. Binding of the Fas ligand to its receptor results in the formation of the DISC at the membrane. This allows for activation of pro-caspase-8 to its active form. The signal can then take one of two pathways. Large amounts of caspase-8 activated at the DISC results in direct activation of effector caspases (e.g. caspase-3) and is independent of the mitochondria (Type I pathway). Small amounts of caspase-8 activated at the DISC require mitochondrial amplification of the apoptotic signal that is mediated through cleavage of Bid. The figure is taken from Nagata, 2000.

(Peter and Krammer, 2003). An adaptor protein known as the Fas associated death domain (FADD) is recruited to the DD and allows for binding of other DISC components such as the imitator caspase pro-caspase-8. This allows for activation of caspase-8 (Peter and Krammer, 2003). Once caspase-8 has been activated it can go on to cleave and activate executioner caspases directly or may cleave other substrates that can amplify the signal through the mitochondrial pathway (Fig. 1-2). One or other of these signalling pathway options is used preferentially, giving two types of cells known as Type I or Type II (Barnhart et al., 2003; Scaffidi et al., 1998; Scaffidi et al., 1999).

In some cell types lots of caspase-8 is activated at the DISC. This results in the direct activation of the executioner caspase caspase-3, thus signalling apoptosis independent of the mitochondrial pathway. These are Type I cells and include the B cell line SKW6.4 and T cell line H9, as well as primary thymocytes (Scaffidi et al., 1999; Yin et al., 1999). However, in other cells there is little caspase-8 activated at the DISC resulting in small amounts of active caspase-3 within the cell. In these cases the weak signal needs to be amplified to ensure that apoptosis progresses efficiently. In such cells the Bcl-2 family member Bid is cleaved by caspase-8 to generate an active C-terminal fragment known as tBid (Li et al., 1998b; Luo et al., 1998). This active form of Bid translocates to the mitochondria, making a connection with the intrinsic death pathway and allows for amplification of the apoptotic signal (Fig. 1-2). These are Type II cells and include the T cell lines CEM and Jurkat, as well as primary hepatocytes (Scaffidi et al., 1999; Yin et al., 1999).

There are also a number of proteins that act as inhibitors of the extrinsic pathway. Within the TNFR family itself are a number of decoy receptors. Expression of decoy receptors can antagonise apoptotic signalling via the extrinsic pathway (Dempsey et al., 2003). There are also a number of adapter proteins that act as inhibitors of apoptotic signalling from the death receptors. Silencer of death domain (SODD) was identified as a protein that inhibited apoptotic signalling from TNF-R1 (TNF receptor 1) by inhibiting the binding of TNF receptor associated death domain (TRADD) and DISC formation (Jiang et al., 1999). In addition, cellular FADD-like interleukin-1β-converting enzyme (FLICE)-inhibitory protein (cFLIP) can also be recruited to the

DISC in Fas signalling. It is homologous to caspase-8 but lacks any caspase activity and can prevent caspase-8 activation (Irmler et al., 1997).

While these receptors appear to function primarily in signalling apoptosis this is not their only function. In a similar way to the formation of the DISC, the recruitment of TNF receptor associated factors (TRAFs) to the receptor results in signalling via a number of pathways (Dempsey et al., 2003). This includes signalling via NF-κB, PI-3K, and p38 ERK/MAPK signalling pathways. Signalling via these pathways regulates many survival, proliferation and differentiation pathways. Thus these 'death receptors' also have roles other than signalling apoptosis.

1.3.2 – The intrinsic pathway

The second predominant apoptotic pathway controlling apoptosis involves the mitochondria. Mitochondria are traditionally known as the powerhouse of the cell and generate the ATP required for normal cellular functioning. However, they also contain a pool of proteins vital for inducing apoptosis. Intrinsic signals that arise within the cell as a result of stress or genotoxic damage converge on the mitochondria to induce apoptosis. Disruption and deregulation of the mitochondria results in the release of these proteins, which promote apoptosis and activation of caspases (Fig. 1-3).

In terms of apoptosis, the Bcl-2 family of proteins regulate mitochondrial integrity (Cory and Adams, 2002; Desagher and Martinou, 2000). Bcl-2 proteins are discussed in greater depth in section 1.4 below. Bax and Bak are two pro-apoptotic members of this family and are instrumental in co-ordinating the release of apoptotic mitochondrial proteins (Wei et al., 2001). Apoptotic signals result in activation of these pro-apoptotic members of the Bcl-2 family and subsequent disruption of the mitochondrial membrane. This allows release of mitochondrial proteins into the cytoplasm, where they function to initiate and promote apoptosis (Desagher and Martinou, 2000). While there is now much known about Bcl-2 family proteins, exactly how they co-ordinate mitochondrial deregulation is not fully understood.

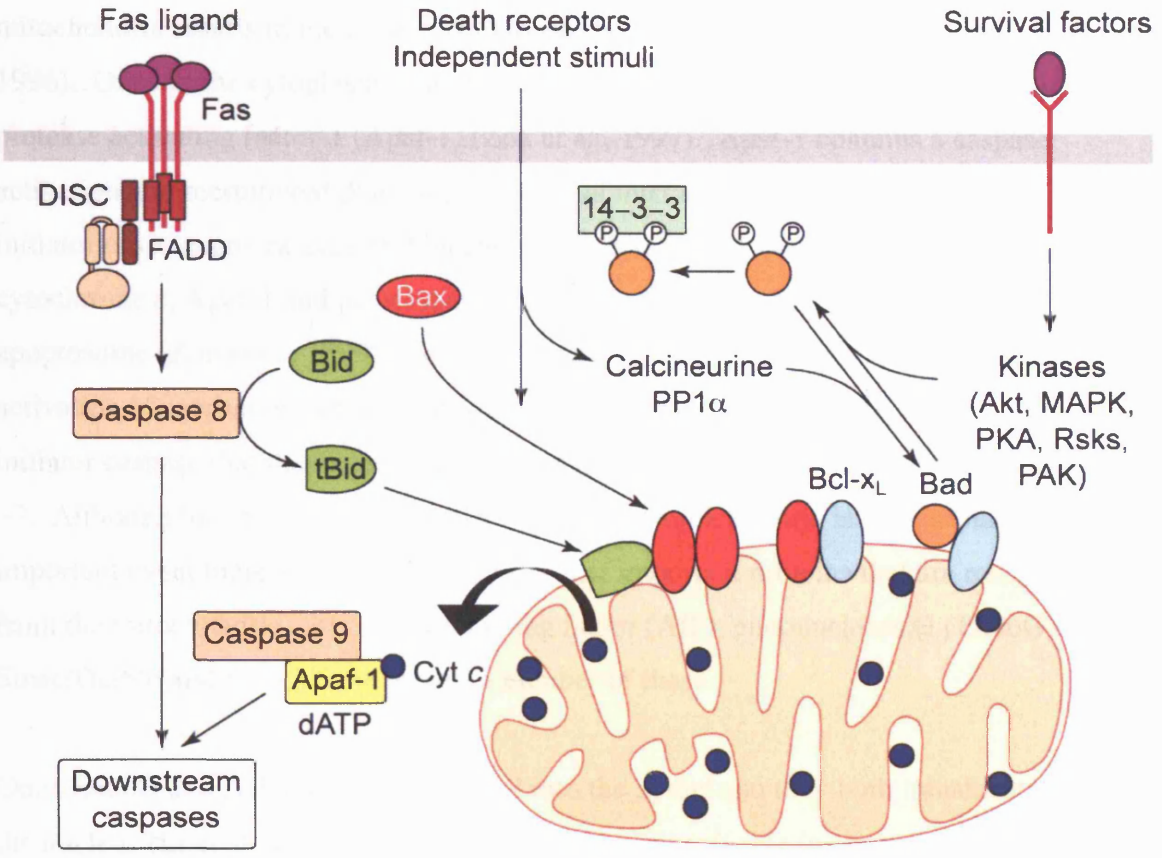


Figure 1-3: Apoptotic signalling via the intrinsic (mitochondrial) pathway. Many apoptotic pathways converge on the mitochondria to induce the release of cytochrome *c* and subsequent activation of caspase-9. Many of these signals activate BH3-only proteins (e.g. Bid or Bad) that then translocate to the mitochondria and result in activation of the pro-apoptotic Bcl-2 family members to induce cytochrome *c* release. Figure taken from Desagher and Martineau, 2000.

Cytochrome *c* is an essential component of the electron transport chain responsible for generation of ATP. During apoptosis it takes on a very different role, one that is critical to signalling apoptosis in the intrinsic death pathway. Deregulation of the mitochondria results in the release of cytochrome *c* into the cytoplasm (Liu et al., 1996). Once in the cytoplasm, cytochrome *c* promotes oligomerisation of apoptotic protease activating factor-1 (Apaf-1) (Zou et al., 1997). Apaf-1 contains a caspase activation and recruitment domain (CARD) that interacts with a similar domain in the initiator caspase pro-caspase-9 (Shiozaki et al., 2002). The complex formed between cytochrome *c*, Apaf-1 and pro-caspase-9, in the presence of dATP, is known as the apoptosome (Zou et al., 1999). Formation of the apoptosome acts as a platform for activation of caspase-9 (Adrain and Martin, 2001; Zou et al., 1999). Caspase-9 is an initiator caspase that then goes on to activate the executioner caspases caspase-3 and –7. Although the release of cytochrome *c* from the mitochondria is an extremely important event there are also a number of other important proteins that are released from the mitochondria. Apoptosis inducing factor (AIF), endonuclease G (EndoG), Smac/Diablo and HtrA2/Omi are just a number of these.

Once EndoG and AIF have been released into the cytoplasm they both translocate to the nucleus (Li et al., 2001; Susin et al., 1999). AIF has a role in chromatin condensation and EndoG is an endonuclease that cleaves DNA inter-nucleosomally. However while they can carry out these functions independent of one another, when expressed together both functions appear to be enhanced (Cregan et al., 2004). In this way they can induce a caspase independent chromatin condensation and nucleosomal cleavage similar to that observed when caspase activated DNase (CAD) is activated (Sakahira et al., 1998).

Proteins such as second mitochondrial activator of caspases (Smac)/DIABLO and HtrA2/Omi also contribute to the activation of caspases (Du et al., 2000; Faccio et al., 2000; Suzuki et al., 2001; Verhagen et al., 2000). They act as inhibitors of the inhibitors of apoptosis (IAP) family of proteins. IAP proteins are endogenous and specific inhibitors of caspases.

IAPs are molecules that bind specifically to caspases and inhibit their activation (Deveraux and Reed, 1999). The first IAP gene was identified in baculovirus where it prevented the infected cell from dying (Crook et al., 1993). All IAPs have one to three baculovirus IAP repeat (BIR) domains and it is these BIR domains that are responsible for the proteins ability to inhibit caspases (Deveraux and Reed, 1999). There are five known mammalian IAP proteins – XIAP, c-IAP1, c-IAP2, NIAP and survivin. XIAP, c-IAP1 and c-IAP2 directly inhibit active caspase-3 and -7 and procaspase-9 with XIAP being the most potent of these inhibitors (Deveraux and Reed, 1999). As well as having a role in inhibiting caspase activity IAPs appear to have other roles in cellular function. In particular, survivin is known to bind microtubules during mitosis, although its BIR domain does not appear to be required for this activity (Li et al., 1998a). Normal cellular functioning has IAPs present in the cytoplasm free to interact with appropriate caspases. Disruption of the mitochondria however results in the release of molecules like SMAC/Diablo and HtrA2/Omi (Du et al., 2000; Faccio et al., 2000; Suzuki et al., 2001; Verhagen et al., 2000). These proteins interact specifically with IAP molecules to relieve inhibition of the caspase proteins allowing them to carry out the function to their full potential.

As the mitochondria represent a key point in the intrinsic pathway of apoptosis its regulation is of key importance. It is likely that there are other mitochondrial proteins that also have roles to play in apoptosis similar to those already discovered. Particularly in the case of the intrinsic pathway, apoptotic signals that converge on the mitochondria arise from various checkpoint proteins that monitor cell growth and viability. Many of these signals require *de novo* expression of proteins and thus transcription and translation of proteins is often required for apoptosis to proceed. Thus there are a number of proteins that direct transcription in order to signal apoptosis to the mitochondria (Kumar and Cakouros, 2004).

1.3.3 – Transcriptional control and activation

Apoptosis is a genetically controlled programmed cell death and as such many apoptotic pathways require changes in gene expression for proper execution of the death program. In particular many of the intrinsic death signals are initiated through changes to gene expression, while signalling from the extrinsic cell death pathway can

also lead to changes in gene expression. Developmental signals, cytokine withdrawal and cytotoxic damage (e.g. UV damage or stress) all elicit a stimulus that requires changes to gene expression.

There are now many transcription factors identified that are known to induce expression of genes involved in protection and activation of apoptosis. These include p53, pRb, E2F-1, c-Jun and NF-κB (Kumar and Cakouros, 2004). These transcription factors do not function to signal apoptosis exclusively but play many roles in the control and regulation of cell growth and proliferation in response to many different stimuli. Through their ability to manipulate the apoptotic program, they are able to orchestrate an appropriate response to eliminate any cells that pose a threat to the organism.

1.3.3.1 – E2F-1 and pRb in apoptosis

The E2F family of proteins (of which E2F-1 is the best studied) and the tumour suppresser protein pRb play important roles in regulation of cell cycle (Cam and Dynlacht, 2003; Stevaux and Dyson, 2002). E2F proteins are known to promote cell growth and proliferation and are controlled through a number of mechanisms including interaction with the tumour suppresser pRb (Dyson, 1998). Overexpression of E2F-1 in cell lines and mouse models can result in apoptosis. E2F-1 can influence apoptosis in a number of ways including inhibition of anti-apoptotic signalling, direct transcriptional activation of p53, or through p53 stabilisation mediated by $p14^{ARF}$ (Phillips and Vousden, 2001). Mice deficient for the *pRb* gene die during embryogenesis (between days E13.5 – E16.5) with extensive apoptosis being one of the causes of death (Lee et al., 1992). This suggests that pRb contributes to survival in normal cells and that loss of the protein results in a death signal. Much of the work in trying to elucidate the molecular mechanism of how the loss of *pRb* induces apoptosis has focused on pRbs ability to interact with E2F. The interaction of pRb with E2F-1 allows for the sequestration of E2F-1, thus preventing it from inducing expression of its target genes. Loss of pRb frees E2F-1 and allows it to transactivate its target genes, inducing apoptosis through either the p53 pathway or through direct induction of genes such as *apaf1* that are directly involved in apoptosis (Phillips and Vousden, 2001). E2F-1 can also transactivate the *p73* gene, a member of the p53

family (Irwin et al., 2000). The p73 protein shares some transcriptional targets with the p53 protein and also plays a role in apoptosis (Melino et al., 2003). As well as inducing expression of pro-apoptotic proteins, E2F-1 is known to repress expression of the anti-apoptotic *mcl-1* gene (Croxton et al., 2002). Thus the pRb and E2F can make a significant contribution to apoptosis in a variety of ways.

1.3.3.2 – p53 signalling in apoptosis

As outlined above, E2F and pRb signal through the p53 protein to induce apoptosis. p53 functions as a critical checkpoint protein, monitoring genome stability and acting as a key decision maker when things go wrong (Okorokov, 2003). Damage to the genome results in a signalling cascade that activates p53 (Pluquet and Hainaut, 2001). Once activated in this manner p53 induces a G1 cell cycle arrest through induction of the cyclin-dependent kinase inhibitor p21 (Dulic et al., 1994). If the damage can be repaired the cell re-enters cell cycle, otherwise p53 can induce a permanent G1 arrest known as premature senescence preventing the cell from dividing any further or it can induce apoptosis. Apoptosis mediated by p53 primarily involves transcription of genes directly involved in apoptosis (Fridman and Lowe, 2003). There are a wide variety of apoptotic genes that can be induced by p53. These include the pro-apoptotic Bcl-2 family members Bax, PUMA and NOXA as well as a host of other proteins including PIG3, p53AIP (p53 apoptosis inducing protein), APAF1 and the CD95 Fas receptor amongst others (Kumar and Cakouros, 2004). The p53 family member p73 has also been found to play a similar role to p53 in apoptosis (Melino et al., 2003). p73 and p53 share activate transcription of number of common targets including Bax, APAF1 and PUMA (Melino et al., 2003). There is also a body of evidence to suggest that some forms of p53 can localise to the mitochondria and may have an apoptotic function that is independent of its transactivation capabilities (Leu et al., 2004; Mihara et al., 2003). p53 that is localised to the mitochondria has been shown to be able to interact with pro-apoptotic Bak resulting in its oligomerisation. This leads to release of cytochrome c and activation of pro-caspase-9 allowing apoptosis to proceed (Leu et al., 2004).

1.3.3.3 – NF-κB and its role in apoptosis

Rel/NF-κB transcription factors play important roles in controlling cell growth and differentiation as well as having a role in apoptosis (Pahl, 1999). There are a number of proteins in the NF-κB family and they bind to their DNA target sites (κB sites) as homodimers or heterodimers. Most of the work on NF-κB relates to its pro-survival function and through its transcriptional activation abilities NF-κB is known to induce expression of many anti-apoptotic proteins including cIAP1, cIAP2, A1, Bcl-X_L and TRAF proteins (Pahl, 1999). However it is clear that NF-κB can also mediate an apoptotic response (Barkett and Gilmore, 1999). Activation-induced cell death (AICD) is an important mechanism for immune cell homeostasis with binding of FasL to its receptor being an important initiation event for AICD. Studies have shown that engagement of the T cell receptor results in upregulation of FasL, mediated by NF-κB suggesting a pro-apoptotic role for NF-κB in AICD (Kasibhatla et al., 1999; Matsui et al., 1998). As well as being able to induce expression of FasL NF-κB is known to induce expression of the Fas receptor (CD95), p53 and the NF-κB inhibitor IκBα which all have a role in inducing apoptosis (Barkett and Gilmore, 1999).

1.3.3.4 – Apoptosis in response to growth factor withdrawal

Cells are constantly receiving survival signals from their environment in the form of cytokines and growth factors. These signals often stimulate expression of anti-apoptotic proteins. For example in haematopoietic cells interleukin-3 (IL-3) stimulates *mcl-1* expression through binding of CREB to a CRE-2 binding site in the *mcl-1* promoter, thus promoting survival of these cells (Wang et al., 1999). Withdrawal of IL-3 results in reduced expression of Mcl-1 and contributes to programmed cell death. Deprivation of IL-3 in pro-B and T cells also results in dephosphorylation of the forkhead transcription factor FKHRL1 and its translocation to the nucleus and transcription of the pro-apoptotic *bim* gene (Dijkers et al., 2000), an important gene required for T cell development (Bouillet et al., 2002; Davey et al., 2002; Hildeman et al., 2002; Villunger et al., 2004). Transcriptional control of *bim* also plays a role in neuronal apoptosis. Sympathetic neurons are dependent on nerve growth factor (NGF) for survival. Removal of NGF results in a signalling cascade

that results activation of c-Jun by increasing levels and phosphorylation of the protein (Ham et al., 1995; Ham et al., 2000). This increase in c-Jun is essential for NGF withdrawal mediated apoptosis and, along with FHKRL1, can activate transcription of the *bim* gene to induce apoptosis (Gilley et al., 2003).

The above examples highlight the importance of controlling transcription during apoptosis. Many of the control and checkpoint proteins that monitor stress within the cell signal apoptosis through proteins such as p53 and activate the intrinsic cell death pathway in this manner. This also emphasises the complexity of programmed cell death in mammals. There are many levels of control required both to initiate apoptosis and also to prevent inappropriate apoptosis.

1.4 – The Bcl-2 family

One of the most important protein families that regulate apoptosis is the Bcl-2 family of proteins. The B cell lymphoma-2 (*bcl-2*) gene was first identified as a potential oncogene as a result of a chromosomal translocation involved in some B-cell lymphomas (Pegoraro et al., 1984). This t(14;18) translocation couples the immunoglobulin heavy chain locus on chromosome 14 to the *bcl-2* locus on chromosome 18, resulting in overexpression of Bcl-2 (Chen-Levy et al., 1989). Initial studies on the function of Bcl-2 identified it as important for cell survival, as distinct from proliferation (Vaux et al., 1988). There have since been a large number of these proteins in this family identified (Fig. 1-4).

The Bcl-2 family of proteins are critical regulators of mitochondrial deregulation during apoptosis (Cory and Adams, 2002; Desagher and Martinou, 2000). Many of the physiological signals and signals arising from intracellular damage that result in apoptosis induce changes to the balance of pro- and anti-apoptotic Bcl-2 family

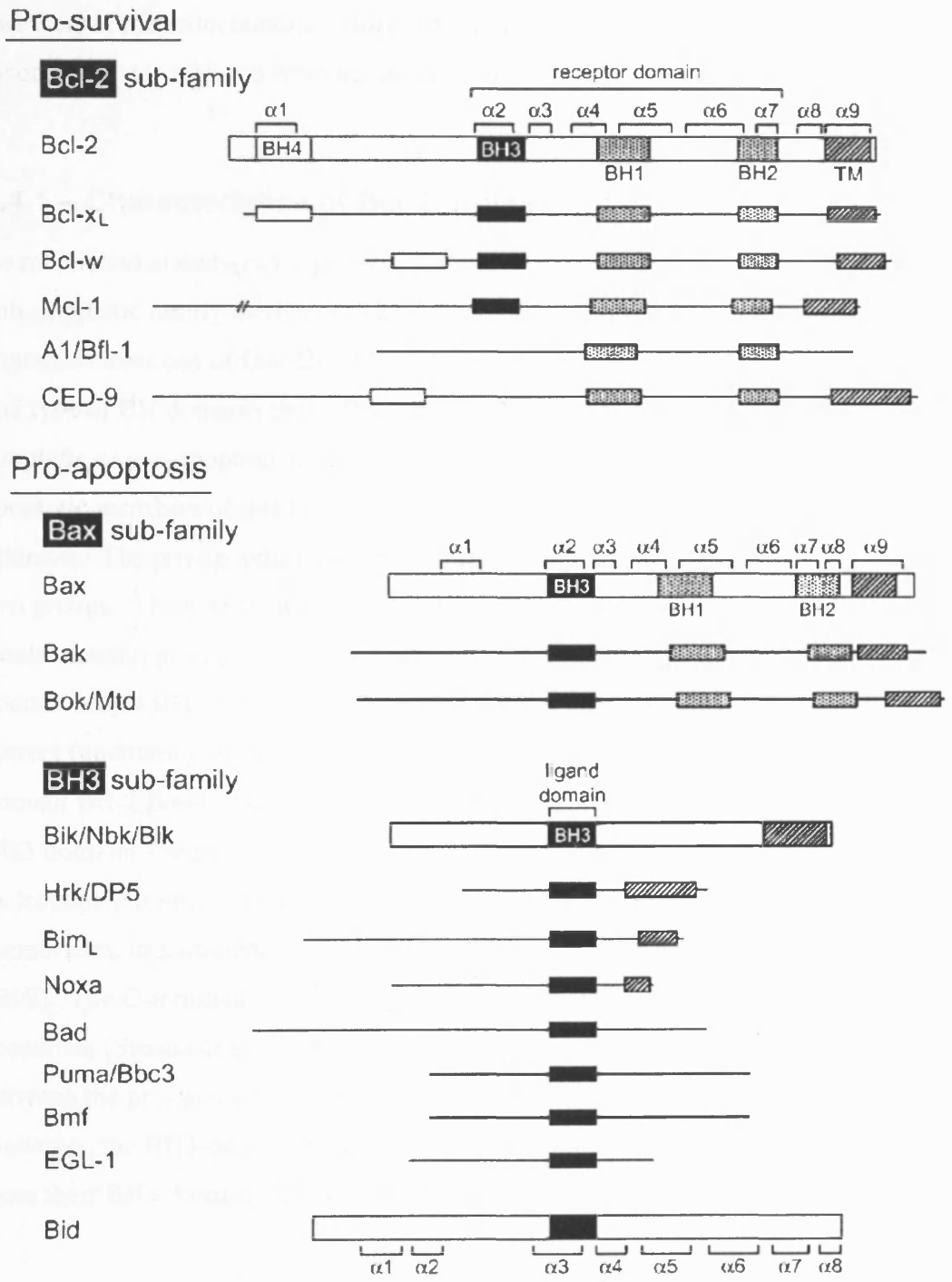


Figure 1-4: Members of the mammalian Bcl-2 family. Bcl-2 proteins represent a large family of proteins that can regulate mitochondrial function during apoptosis. The role of many of these proteins remains to be further clarified from a physiological point of view. Figure taken from Cory et al., 2003.

members at the mitochondria. Once the pro-apoptotic members take control cytochrome c is released from the mitochondria and apoptosis progresses.

1.4.1 – Characteristics of Bcl-2 proteins

As mentioned already Bcl-2 proteins can be divided into two main groups, pro- and anti-apoptotic family members. The defining feature of all Bcl-2 proteins is that they contain at least one of four Bcl-2 homology domains (BH1-4) (Fig. 1-4). The number and type of BH domains that a Bcl-2 protein has determines whether it is an anti-apoptotic or pro-apoptotic protein (Cory and Adams, 2002). Many of the anti-apoptotic members of this family (e.g. Bcl-2 and Bcl-X_L) contain all four BH domains. The pro-apoptotic members of the family can be further sub-divided into two groups. Those such as Bax and Bak that contain BH1, BH2 and BH3 domains (multi-domain pro-apoptotic Bcl-2 proteins), or those such as Bid, Bim, Bad, etc that contain only a BH3 domain (BH3-only proteins). The BH domains are critical for correct functioning of these proteins. The BH1, BH2 and BH3 domains of the multi-domain Bcl-2 family members form a hydrophobic cleft that acts as an acceptor for BH3 domains (Sattler et al., 1997). Many of these proteins contain a C-terminal hydrophobic transmembrane domain that allows for insertion into intracellular membranes, in particular to the outer membrane of the mitochondria (Goping et al., 1998). The C-terminal domain of Bax also affects its conformation and dimerisation properties (Suzuki et al., 2000). It is interesting to note that there is high homology between the pro- and anti-apoptotic multi-domain members of the Bcl-2 family. However, the BH3-only proteins share little homology with other Bcl-2 proteins apart from their BH3 domain (Kelekar and Thompson, 1998).

1.4.2 – Bcl-2 proteins and the mitochondrion

The manner in which Bcl-2 family members co-ordinate mitochondrial deregulation and cytochrome *c* release is not very well understood. Overexpression of the anti-apoptotic Bcl-2 protein can completely block cytochrome *c* release in some cell types (Kluck et al., 1997; Yang et al., 1997) and cell-free systems suggest that Bcl-2 must be associated with the mitochondria to prevent cytochrome *c* release (Kluck et al., 1997). Conversely, the overexpression of Bax in some cells is sufficient to initiate

apoptosis in the absence of a death stimulus (Jurgensmeier et al., 1998; Rosse et al., 1998). However the mechanism by which Bcl-2 family members control this is poorly understood. There are a number of possible models for how cytochrome *c* is released from mitochondria during apoptosis (Desagher and Martinou, 2000). Some of these models are based on swelling of the mitochondrial matrix during early apoptosis resulting in rupture and release of mitochondrial proteins into the cytoplasm. Evidence for such models is scant and as there appears to be a selective release of mitochondrial proteins into the cytoplasm this does not fit with these models.

A more attractive model is the release of cytochrome *c* from the mitochondria through pore formation in the outer membrane. The three dimensional structures of Bcl-2 family members have been shown to be similar to the pore forming domains of diphtheria toxin and some bacterial colicins (Muchmore et al., 1996). Many of the Bcl-2 family proteins have also been shown to form pores in synthetic lipid vesicles and planar lipid bilayers (Schendel et al., 1999; Schendel et al., 1998). Although the helical domains of Bcl-2 family members are not large enough themselves to span the outer mitochondrial membrane, Bax and Bak oligomerise during apoptosis and hence could potentially form much larger channels through which mitochondrial proteins may be exported into the cytoplasm. Indeed this oligomerisation of these pro-apoptotic proteins appears to be required for cytochrome *c* release and is inhibited by anti-apoptotic proteins such Bcl-2 and Bcl-X_L. While the Bax or Bak may themselves have channel forming capabilities there is also the possibility that they may interact with other channel proteins present in the outer mitochondrial membrane to release cytochrome *c*. Bax is known to interact with the voltage dependent anion channel (VDAC) and may co-ordinate cytochrome *c* release by controlling this channel in some manner (Cheng et al., 2003). Finally there is also the possibility that the Bcl-2 family of proteins may form a lipidic pore or protein–lipid complex to allow mitochondrial proteins to diffuse into the cytosol. Much of the evidence for the ability of Bcl-2 proteins to form pores and channels comes from *in vitro* studies and so it is difficult to know if they could form such pores and channels *in vivo* (Desagher and Martinou, 2000).

1.4.3 – BH3-only proteins

The number of BH3-only members of the Bcl-2 family represents a large proportion of the members of this family. Apart from their ~9 amino acid BH3 domain, they share little homology with each other or with other members of the Bcl-2 family (Kelekar and Thompson, 1998). Many of these BH3-only proteins sequestered within the cytoplasm in an inactive form and are activated quickly in response to an apoptotic stimulus. Once activated they translocate to the mitochondria where they interact with other Bcl-2 proteins to promote apoptosis through Bax and Bak activation. These proteins are often the first Bcl-2 proteins activated in response to specific apoptotic signals. Studies of BH3 domain peptides have identified two mechanisms through which these BH3-only proteins appear to work (Chittenden, 2002; Letai et al., 2002). These mechanisms are based on the observation that BH3 domain peptides derived from different BH3-only proteins have different specificities for the multi-domain Bcl-2 family members. BH3 peptides derived from Bid or Bim BH3 domains can bind to both the pro-apoptotic Bax and Bak, and to anti-apoptotic Bcl-2 (Letai et al., 2002). On the other hand, BH3 peptides derived from Bad and Bik appear to only bind to anti-apoptotic Bcl-2 (Letai et al., 2002). Thus Bid or Bim may act as *"activator"* proteins, by directly activating Bax and Bak. In this setting anti-apoptotic proteins such as Bcl-2 and Bcl-X_L act like decoy receptors sequestering the BH3-only protein and suppressing the signal. Bad and Bik may act as *"enabler"* proteins, interacting with anti-apoptotic Bcl-2, and allowing for easy activation of Bax and Bak (Chittenden, 2002; Letai et al., 2002). This is illustrated in Figure 1-5.

1.4.4 – Regulation of BH3-only proteins

Some BH3-only proteins are regulated at a transcriptional level and are quickly upregulated in response to a death stimulus (Kumar and Cakouros, 2004). However most of the BH3-only proteins exist in inactive forms within the cell with activation of these proteins carried out through various post-translational modifications (Kelekar and Thompson, 1998).

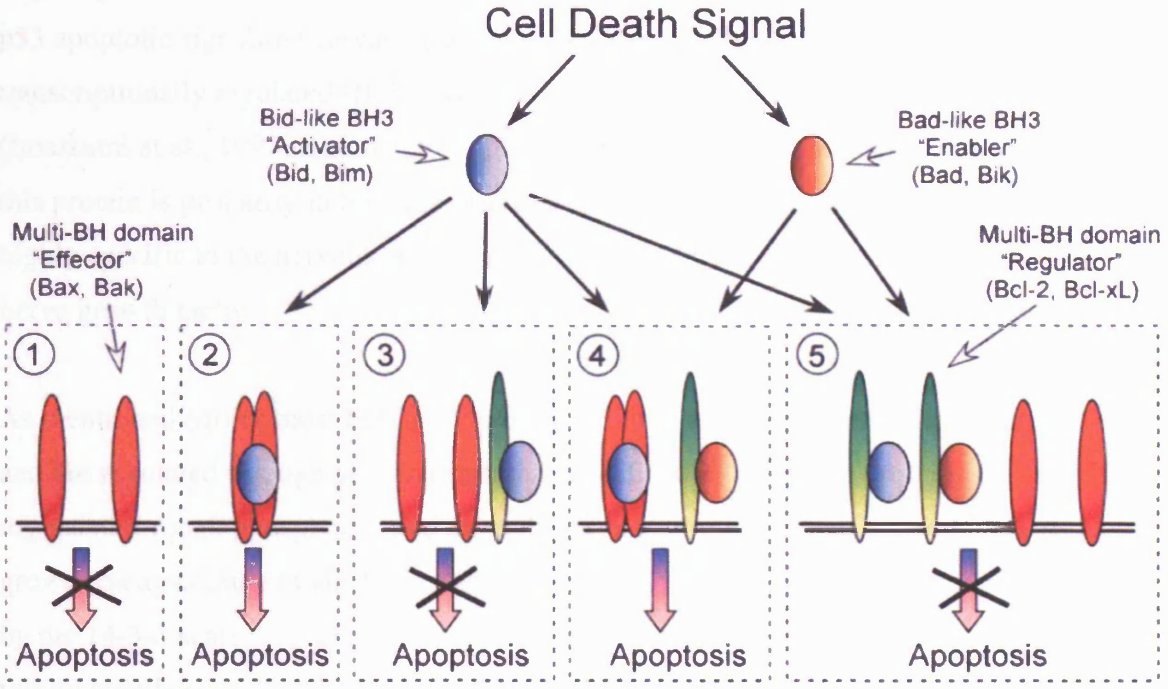


Figure 1-5: A model for the control of apoptosis by BH3-only proteins. The pro-apoptotic multi-domain proteins Bax and Bak normally exist in an inactive state at the mitochondria (1). Some BH3-only proteins may contain an activating (or effector) BH3 domain (e.g. Bid and Bim) that can directly activate and induce oligomerisation of Bax/Bak (2). In this case Bcl-2 anti-apoptotic proteins function to sequester the activating BH3-only protein and prevent activation of Bax/Bak (3). Other BH3-only proteins may contain a sensitizing (or enabler) BH3 domain that interacts with anti-apoptotic Bcl-2 proteins and sensitizing the cell to the effects of activating BH3-only proteins (4). Over-expression of anti-apoptotic Bcl-2 family members can lead to the sequestration of both types of BH3-only proteins, thus blocking apoptosis completely (5). Figure taken from Chittenden, 2002.

The BH3-only proteins PUMA and Noxa have been shown to be direct transcriptional targets of the p53 protein (Nakano and Vousden, 2001; Oda et al., 2000). Gene targeting studies in mice have shown these proteins to be important mediators of the p53 apoptotic signalling cascade (Jeffers et al., 2003; Shibue et al., 2003). Another transcriptionally regulated BH3-only protein is the neuronal specific DP5/Hrk (Imaizumi et al., 1997; Inohara et al., 1997). Expression of the human homologue of this protein is primarily in lymphoid tissue while DP5, the rodent homologue, is highly specific to the nervous system and contributes to apoptosis in the absence of nerve growth factor.

As mentioned earlier most BH3 only proteins exist in an inactive form within the cell and are regulated through post-translational modification. The Bad protein is regulated through phosphorylation on multiple sites through signalling from various growth factors (Datta et al., 1997; del Peso et al., 1997). Phospho-Bad is sequestered by the 14-3-3 scaffold proteins and so does not come into contact with other Bcl-2 family members at the mitochondria (Zha et al., 1996). Bim is also sequestered in the cell although through binding to the dynein light chain LC8, which is part of the motor dynein complex (Zha et al., 1996). In response to lack of survival signals or apoptotic signals Bad and Bim are released from their sequestration partners and translocate to the mitochondria where they induce cytochrome *c* release (Kelekar and Thompson, 1998). Bid, a molecule we are particularly interested in, also exists in an inactive form (p22) within the cell. Signalling via death receptors results in activation of caspase-8, which goes on to cleave Bid to generate an active C terminal fragment (p15) known as truncated Bid (tBid) (Li et al., 1998b; Luo et al., 1998). This active form of the Bid protein then translocates to the mitochondria to induce mitochondrial deregulation (Korsmeyer et al., 2000; Wei et al., 2000).

Thus it is likely that these BH3-only proteins act as sensors within the cell to detect damage and signals that requires apoptosis and are critical initiators of mitochondrial deregulation and cytochrome *c* release.

1.5. – Bid

1.5.1 – Identification of Bid

Bid (*B*H3 *i*nteracting domain *d*eath agonist) was initially cloned through an interactive cloning technique that identified proteins that interact with both Bcl-2 and Bax (Wang et al., 1996). A cDNA encoding the Bid protein was identified in a number of clones and a BLAST search identified a region sharing high homology with a BH3 domain. Apart from its BH3 domain Bid did not appear to share any homology with any other Bcl-2 family member. The protein was found to be expressed in adult mouse brain, spleen, liver and testis with a very high expression level in kidney. There appeared to be little expression of the protein in heart or skeletal muscle. There did not appear to be any hydrophobic transmembrane domain in the protein as is the case with other Bcl-2 family members and cell fractionation experiments confirmed the proteins mainly cytosolic localisation (Wang et al., 1996).

1.5.2 – Regulation of Bid

Upon induction of apoptosis via the death receptor pathway, formation of a death induced signalling complex results in activation of initiator caspases such as caspase-8 (Peter and Krammer, 2003). Two studies examining how caspase-8 mediates mitochondrial damage identified Bid as a substrate for the enzyme (Li et al., 1998b; Luo et al., 1998). Cleavage of Bid by caspase-8 generates a truncated active C terminal fragment called tBid. This active tBid fragment translocates to the mitochondria where it induces cytochrome *c* release resulting in formation of the apoptosome and subsequent caspse-9 activation (Adrain and Martin, 2001; Korsmeyer et al., 2000). Thus tBid acts to amplify the signal from the death receptor. As well as being cleaved by caspase-8 Bid is also cleaved by granzyme B to generate gtBid, a 14-kDa fragment similar to that generated by caspase-8 cleavage (Barry et al., 2000; Heibein et al., 2000). Granzyme B is a serine protease that cleaves substrates after aspartate residues and is involved in death induced by cytotoxic T lymphocytes (CTL) (Lord et al., 2003). CTLs can kill target cells through the release of granules containing the pore forming protein perforin and granzymes.

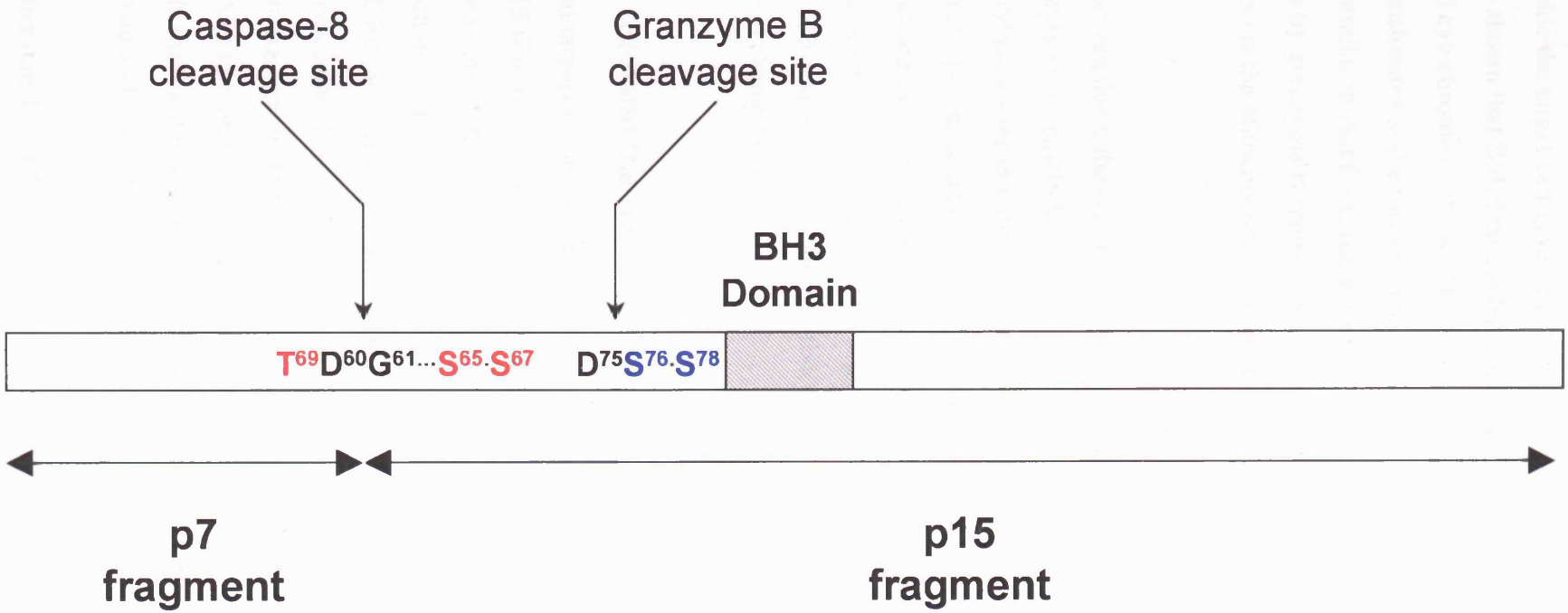


Figure 1-6: The BH3-only protein Bid. The schematic shows the caspase-8 and granzyme B cleavage sites at Asp^{60} and Asp^{75} respectively for human Bid. Also highlighted is the BH3 domain. This is a 9 amino acid stretch from Leu^{90} to Asp^{98}. Sites of major phosphorylation are in red, with likely minor sites of phosphorylation in blue.

Once inside the target cell granzyme B can cleave caspase-3, caspase-8 and Bid. It has been shown that Bid cleavage by granzyme B is required for granzyme B-mediated cytochrome *c* release (Heibein et al., 2000). Once cleaved by granzyme B gtBid translocates to the mitochondria where it mediates cytochrome c release in a manner similar to that for caspase cleaved tBid. This mechanism of apoptotic initiation by granzyme B represents an important pathway by which CTLs can induce apoptosis via the mitochondria without the initial activation of caspases (Lord et al., 2003).

Bid is also regulated through phosphorylation (Desagher et al., 2001). It is phosphorylated by casein kinases I and II. There appears to be a number of phosphorylation sites in mouse Bid at T58, S61 and S64. Other phosphorylation sites may also exist close to this region. This phosphorylation of Bid is likely to be sequential and can prevent cleavage by caspase-8 (Desagher et al., 2001). Bid has also been reported to be regulated through p53 (Sax et al., 2002). In this situation *bid* acts as a p53-responsive 'chemosensitivity gene' that may enhance the cell death response to chemotherapy.

1.5.3 – tBid and the mitochondrion

How tBid targets the mitochondria is unclear. A post-translational N-myristoylation of the p15 tBid fragment appears to be a factor in the rapid targeting of the protein to the mitochondria (Zha et al., 2000). There is also evidence that tBid translocation to the mitochondria is mediated by the mitochondrial lipid cardiolipin (Lutter et al., 2000). Cardiolipin is only found in the mitochondrial inner membrane and at inter-membrane contact points (McMillin and Dowhan, 2002). The binding of tBid to the mitochondria appears to be dependent on the amount of cardiolipin in the membrane as tBid does not interact directly with cardiolipin *in vitro* (Lutter et al., 2000). Thus it is thought that cardiolipin may effect mitochondrial membrane structure allowing tBid to bind and integrate into the membrane (McMillin and Dowhan, 2002).

Once it has translocated to the mitochondria tBid carries out its apoptotic function. The BH3 domain of Bid is known to interact with other pro- and anti-apoptotic Bcl-2 proteins (Letai et al., 2002). In particular Bid is thought to have an 'activating'

function through its ability to induce homo-oligomerisation, and hence activation, of Bax and Bak (Desagher et al., 1999; Eskes et al., 2000; Korsmeyer et al., 2000; Roucou et al., 2002; Wei et al., 2000). As Bid can also interact with anti-apoptotic Bcl-2 family members it is thought that they function to sequester tBid and prevent it from activating the pro-apoptotic Bax and Bak (Cheng et al., 2001). Although tBid has been shown to be capable of forming ion channels *in vitro* it is not clear if this happens *in vivo* (Schendel et al., 1999). It has also been shown that Bax and tBid can decrease the stability of planar phospholipid bilayers and thus suggesting that perhaps these pro-apoptotic proteins may destabilise the outer mitochondrial membrane (Basanez et al., 1999; Kudla et al., 2000). In this way they could perhaps induce release of mitochondrial proteins through the through the formation of a lipidic pore or protein lipid complex (Desagher and Martinou, 2000). However, exactly how tBid activates and induces mitochondrial deregulation and cytochrome *c* release through Bax and Bak is unclear, although it is critical for its function. Bid mediated cytochrome *c* release results in formation of the apoptosome and activation of caspase-9 (Adrain and Martin, 2001). This is an important mechanism for amplification of apoptotic signals to ensure efficient and complete apoptosis.

1.5.4 – The physiological significance of Bid

Gene targeting studies to generate Bid-deficient mice showed no apparent developmental abnormalities with mice born completely normal and at the normal mendelian frequency (Yin et al., 1999). As Bid is cleaved in response to DISC formation, mice were injected with the activating anti-Fas (Jo2) antibody to activate this pathway in mice. Most of the wild-type mice injected with the antibody died quickly from massive hepatic apoptosis and haemorrhaging of the liver. Of the Bid-deficient mice injected with the antibody most survived the insult and had no apparent damage to the liver (Yin et al., 1999). Culturing of mouse embryo fibroblasts (MEF) and thymocytes from targeted mice showed an impaired apoptotic response to (Bid mediated) Fas signalling and little effect from other stimuli (Yin et al., 1999). Thus while some cellular systems may depend on Bid to signal apoptosis in response to Fas signalling many cellular systems are not dependent on Bid for progression of apoptosis, but use the ability of Bid to amplify the apoptotic signal through cytochrome *c* release. Follow-up studies on the Bid deficient mice showed an altered

myeloid homeostasis with an elevated absolute neutrophil count accompanied by mild hepatosplenomegaly (Zinkel et al., 2003). While haematopoietic cell populations in Bid-/- mice of under 12 months of age appeared normal the bone marrow of young (6 to 12 weeks of age) mice demonstrated increased colony-forming units of macrophage and mixed colonies in methylcellulose (Zinkel et al., 2003). As the Bid-/- mice age they spontaneously develop a myeloproliferative disorder, which progresses from myeloid hyperplasia to a clonal malignancy resembling chronic myelomonocytic leukemia (CMML). This shows that Bid plays an important role in a number of tissues. It is of critical importance for Fas induced apoptosis in hepatocytes and an important amplifier of this signal in other cell types. In addition it is critical in the regulation of normal myeloid homeostasis and for suppression of leukaemias of this lineage.

1.6 – Project aims

The initial aim of this project were to identify novel interactions with the pro-apoptotic tBid protein in order to further elucidate and clarify how the protein carries out its function. We decided to carry out a yeast two-hybrid assay to identify such novel interactions.

2 - Materials and Methods

2.1 – Materials

2.1.1 – Reagents

All media and cell culture reagents were obtained from Gibco. All other reagents were purchased from Sigma unless otherwise stated. All primers for PCR reactions are given in Appendix I.

2.1.2 – *E.coli* strains used

BL21(DE3) F⁻ *omp*T *gal [dcm] [lon] hsd*S_B ($r_B^- m_B^-$; an *E. coli* B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene

DH10B F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*Z74 *deo*R *rec*A1 *end*A1 *ara*Δ139 Δ(*ara, leu*)7697 *gal*U *gal*K λ⁻ *rps*L (Str^R)

2.1.3 – Yeast strain used

Y190 (*MATa, ura3-52, his3-Δ200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, URA3::*GAL_{UAS}*-GAL1*$_{TATA}$*-lacZ, cyh*^{r}*2, LYS2::GAL1*$_{UAS}$*-HIS3*$_{TATA}$*-HIS3*)

2.1.4 – Cell lines

Jurkat human T-cell line (ECACC)

LinXE virus packaging cell line

FDCP-1 murine myeloid progenitor cell lines (ATCC)

2.1.5 – Antibodies

Name	Source	Isotype	Working Concentration	Origin
Bax, NT	Rabbit	IgG	WB – 1:1000	Upstate
Bid (M-20)	Rabbit	IgG	WB – 1:1000	Santa Cruz
Bid	Goat	IgG	WB – 1:1000	RnD Systems
Gal-4 (Gal4-8)	Mouse	$IgG_{1\kappa}$	WB – 1:1000	Zymed
HA (3F10)	Rat	IgG_1	WB – 1:1500	Roche
Mcl-1 (sc-19)	Rabbit	IgG	WB – 1:500	Santa Cruz
PARP	Mouse	IgG_1	WB – 1:1000	BD Pharmingen
Tubulin (YL1/2)	Rat	IgG_{2a}	WB – 1:5000	Serotec

For Western blotting (WB) all antibodies were diluted in TBS-T containing 5% non-fat milk (Marvel).

2.1.6 – Media for culture of *E. coli* bacteria

LB broth — 1.0% (w/v) tryptone, 0.5% (w/v) yeast extract, 1.0% (w/v) NaCl (pH 7.0)

LB agar — LB broth medium plus 15 g/l agar

Psi broth — 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.4% (w/v) $MgSO_4$, 10 mM KCl, adjust pH to 7.6 with 1 M KOH

2.1.7 – Media for culture of Y190 yeast

YEP	10 g/l yeast extract (Difco), 20 g/l Bacto Peptone (Difco)
YPD	YEP with 4% (w/v) glucose
-WHAUL Dropout Broth	0.12 mM L-arginine, 0.8 mM L-aspartic acid, 0.7 mM L-glutamic acid, 0.14 mM L-lysine•HCl, 0.14 mM L-methionine, 0.3 mM L-phenylalanine, 3.65 mM L-serine, 1.7 mM L-threonine, 0.17 mM L-tyrosine, and 1.3 mM L-valine to pH 5.4 with NaOH
-WHAUL Agar	-WHAUL broth with 23.2 g/l agar (Difco)
40 % Glucose	400 g/l D-glucose filter-sterilised
10 × Yeast Nitrogen Base (YNB)	67 g/l yeast nitrogen base (Difco) filter sterilised
-UW media	-WHAUL broth/agar with 0.23 mM L-histidine, 0.8 mM L-leucine, 1 mM adenine, 2% (w/v) glucose and 1 × YNB
-WL media	-WHAUL broth/agar with 0.23 mM L-histidine, 0.32 mM uracil, 1 mM adenine, 2% (w/v) glucose and 1 × YNB
-HWL + 3AT media	-WHAUL broth/agar with 0.32 mM uracil, 1 mM adenine, 17.5 mM 3-amino triazole (3-AT), 2% (w/v) glucose and 1 × YNB

2.1.8 – Buffers and solutions

DNA loading buffer (6 ×)	0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF and 30% (w/v) glycerol in water
Fixing solution	50% (v/v) methanol, 10% (v/v) glacial acetic acid
10 × Lithium Acetate	1 M lithium acetate to pH 7.5 with dilute acetic acid
Lysis Buffer	50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40. To this was added 1 tablet of complete inhibitors (Roche) per 10 ml buffer just prior to use
PIPES Buffer	50 mM PIPES/KOH (pH 6.5), 2 mM EDTA, 0.1% (w/v) CHAPS and 1 tablet of complete inhibitors (Roche) per 10 ml of buffer
Protein running Buffer	25 mM Tris-HCl (pH 8.3), 192 mM glycine, 0.1% (w/v) SDS
Protein Transfer Buffer	12 mM Tris-HCl (pH 8.3), 96 mM glycine, 20% (v/v) methanol
Sample Buffer (2 ×)	100 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 1% (w/v) bromophenol blue, 20% (w/v) glycerol, 200 mM DTT
TAE	40 mM Tris acetate, 1 mM EDTA

TBS	20 mM Tris-HCl pH 7.5, 150 mM NaCl
TBS-T	TBS with 0.1% (v/v) Tween
TE	10 mM Tris-HCl, 1 mM EDTA to pH 7.5
10 × TE	100 mM Tris-HCl, 10 mM EDTA to pH 7.5
TFBI	100 mM $RbCl_2$, 50 mM $MnCl_2$, 30 mM KOAc, 10 mM $CaCl_2$, 15% (v/v) glycerol, pH to 5.8 with 0.2 M AcOH
TFBII	10 mM MOPS pH 7.0 (adjust with NaOH), 10 mM $RbCl_2$, 75 mM $CaCl_2$, 15% (v/v) glycerol

2.2 – Methods

2.2.1 – Molecular biology

2.2.1.1 – Polymerase chain reaction (PCR)

PCR was used to amplify up many of the cDNAs before cloning them into the appropriate vectors. Unless otherwise stated, the reaction mixtures used contained 1× *Pfu* polymerase buffer (containing 1.5 mM $MgCl_2$) (Promega), 200 μM dNTPs (Promega), 100 ng of appropriate primers and 5 units of *Pfu* polymerase (Promega). Water was added to bring the final volume to 50 μl. Conditions used for PCR were: 1 cycle of 94°C for 1 min 30 sec; 30 cycles of 94°C 1 min, 57 - 62°C (depending on the cDNA being amplified) for 1 min, 72°C for 2 min; 1 cycle of 72°C for 5 min. PCR products were analysed by gel electrophoresis using a 1% (w/v) TAE-agarose gel containing 0.5 μg/ml ethidium bromide and viewed using a UV transilluminator.

2.2.1.2 – RT-PCR analysis of Mcl-1 constructs

RNA was prepared from 3×10^6 cells for each of non-transduced FDCP-1 cells, or FDCP-1 cells transduced with pMSCV- HA-Mcl-1-IRES-huCD2, pMSCV-HA-Δ127-Mcl-1-IRES-huCD2, or pMSCV-HA-Δ157-Mcl-1-IRES-huCD2 using a Qiagen RNeasy mini kit, as per the manufacturers instructions. Reverse transcription was carried out using an oligo-dT primer and Superscript II reverse transcriptase (Invitrogen) according to the manufacturers instructions. The resulting cDNA was used for PCR to amplify β-Actin using the primer pair Actin F3 and Actin B3; while a C-terminal region from overexpressed Mcl-1 constructs was amplified using the primer pair Mcl-1-3F and pcDNA3.1-R.

2.2.1.3 – Constructs for yeast two-hybrid assay

A mouse *tbid* cDNA was amplified by PCR from a murine pcDNA3-Bid template (gift from Dr. S. Korsmeyer). The primers used for PCR were 5´mtBid-*Eco*R I and

3´mBid-*Bam*H I. The PCR product was cleaned up using a Qiagen PCR clean-up kit as per the manufacturers instructions. This was then digested using *Eco*R I and *Bam*H I and again cleaned up using a Qiagen PCR clean-up kit. The bait vector pGBKT7 (Clontech) was prepared by digesting it with *Eco*R I and *Bam*H I, and subsequently treating it with calf intestinal phosphatase (CIP) (Roche) according to manufacturers instructions. The vector DNA was cleaned up using a Qiagen PCR clean-up kit. The *tbid* insert was ligated into the pGBKT7 vector in a molar ratio of 1:5 (vector:insert). Ligation reactions were carried out in 10 µl volumes using T4 DNA ligase (Promega) according to the manufactures instructions.

Mouse *baxα* lacking the C terminal hydrophobic domain (*baxΔ21*) was cloned by PCR from a pcDNA3-Baxα template. The primers used for PCR were 5´BaxΔ21-*Eco*R I and 3´BaxΔ21-*Cla* I. The PCR product was cleaned up using a Qiagen PCR clean-up kit as per the manufacturers instructions. This was then digested using *Eco*R I and *Cla* I and again cleaned up using a Qiagen PCR clean-up kit. The prey vector pGADT7 (Clontech) was prepared by digesting it with *Eco*R I and *Cla* I, and subsequently treating it with CIP according to manufacturers instructions. The vector DNA was cleaned up using a Qiagen PCR clean-up kit. Ligations were carried out as outlined above.

The mouse cDNA library used for the yeast two-hybrid assay was a kind gift from Dr. Gabriel Gil-Gómez. Briefly, mouse (FVB) thymocytes were irradiated with 5 Gy and cultured for 5 hours. The RNA was then purified from these cells. A cDNA library was generated by random priming and was directionally cloned in the *Eco*R I – *Xho* I sites of the prey vector pAD-Gal4-2.1 (Stratagene). The library was amplified once and found to have over 90% recombinants with an average insert size of 1.5 kb.

2.2.1.4 – Generation of a pcDNA3.1-HA vector

The plasmid vector pcDNA3.1-HA was used for all *in vitro* translation assays and transient transfection experiments. This was generated by first annealing the two oligos 5´-
TCGAGACCATGGCTTATCCTTATGACGTGCCTGACTATGCCAGCCTGG-3´

and 5´-
AATTCCAGGCTGGCATAGTCAGGCACGTCATAAGGATAAGCCATGGTC-3´.
This generates the following double stranded oligo –

```
5´TCGAGACCATGGCTTATCCTTATGACGTGCCTGACTATGCCAGCCTGG      3´
      ||||||||||||||||||||||||||||||||||||||||||||||||
3´    CTGGTACCGAATAGGAATACTGCACGGACTGATACGGTCGGACCTTAA 5´
```

with *Xho* I and *Eco*RI overhangs, which was then ligated into the *Xho* I and *Eco*RI sites of pcDNA3.1/myc-HIS©(-)B (Invitrogen).

2.2.1.5 – Cloning of human cDNAs into pcDNA3.1 vectors

A pUC18-*bcl2* (Clonexpress) construct was used to clone the human *bcl-2* cDNA. This construct was digested with *Eco*R I and *Hin*d III to release the *bcl-2* cDNA. This was purified by running the sample out on a 1% agarose gel and extracting the cDNA from the gel using a Qiagen gel-extraction kit, according to the manufacturers instructions. The cDNA was ligated into pcDNA3.1/myc-HIS©(-)B that had been pre-digested *Eco*R I and *Hin*d III.

All of the remaining human cDNA constructs were cloned into the *Eco*R I – *Bam*H I sites of pcDNA3.1-HA such that they were in the same open reading frame as the HA epitope tag.

Human *bid* and *tbid* cDNA was generated by PCR from oligo-dT primed cDNA obtained from Jurkat T cells (gift from Dr. J. Zhuang). The primers used to amplify *bid* were 5´hBid-*Eco*R I and 3´hBid-*Bam*H I. For amplification of *tbid* the primers 5´htBid-*Eco*R I and 3´hBid-*Bam*H I were used.

The human *ndpk-b* cDNA was amplified by PCR from Jurkat cDNA as above. The primers used were 5´hNM23B-*Eco*R I and 3´hNM23B-*Bam*H I.

The human *eid-1* cDNA was amplified by PCR from a pcDNA3-T7-EID-1 template (a gift from Dr. W.G. Kaelin Jr.). The primers used were 5´hEID-1-*Eco*R I and 3´hEID-1-*Bam*H I.

Human *cdc37* cDNA was amplified from a pcDNA3-CDC37 template (a gift from Dr. J.W. Harper) using the primers 5´hCDC37-*Eco*R I and 3´CDC37-*Bam*H I.

Both mouse and human *mcl-1* were amplified by PCR from pcDNA3-EAT (gift from Dr. J. Hata) and pBluescript-Mcl-1 (gift from Dr. S. Edwards) respectively. The primers used for PCR were 5´Mcl-1-*Eco*R I and 3´Mcl-1-*Bam*H I. The caspase cleavage fragments of Mcl-1 were also cloned by PCR into the *Eco*RI and *Bam*HI sites of pcDNA3.1-HA. 127-Mcl-1 was amplified using the primer pair 5'Mcl-1-*Eco*R I and 3´Mcl-127-R, Δ127-Mcl-1 was amplified with 5´Mcl-Δ127-F and 3'Mcl-1-*Bam*HI, and Δ157-Mcl-1 was amplified using the primers 5´Mcl-Δ157-F and 3'Mcl-1-*Bam*H I.

2.2.1.6 – Site directed mutagenesis

Mutant *mcl-1* constructs D127A-Mcl-1 and D157A-Mcl-1 were generated by site directed mutagenesis using the GeneTailor™ Site-Directed Mutagenesis System from Invitrogen, according to the manufacturers instructions. The primer pairs used for D127A-Mcl-1 were D127A-F and D127A-R. The primer pairs used for D157A-MCL-1 were D157A-F and D157A-R. A double mutant molecule (DblMut-Mcl-1) was generated using the D127A-Mcl-1 as a template and mutating Asp^{157} to Ala using the primers D157A-F and D157A-R as before.

2.2.1.7 – Constructs for production of GST-fusion proteins

The vector used to generate GST-fusion proteins was pGEX-6P-2 (Amersham). Both *bid* and *tbid* cDNAs were cloned by PCR into the *Eco*R I and *Xho* I sites of the multiple cloning site, while *mcl-1* and *eid-1* cDNAs were cloned into the *Bam*H I and *Eco*R I sites in the multiple cloning site. All cDNAs were cloned so as to be in the same open reading frame as the upstream GST protein.

Using pcDNA3.1-HA-Bid as a template, the primers 5′GST-Bid-*Eco*R I and 3′GST-Bid-*Xho* I were used to PCR the *bid* cDNA for cloning. For *tbid* the primers used were 5′GST-tBid-*Eco*R I and 3′GST-Bid-*Xho* I.

Primers used to amplify *mcl-1* from pcDNA3.1-HA-Mcl-1 were 5′GST-Mcl-1-*Bam*H I and 3′GST-Mcl-1-*Eco*R I. For *eid-1* primers used were 5′GST-EID-1- *Bam*H I and 3′GST-EID-1- *Eco*R I, and again the template used was pcDAN3.1-HA-EID-1.

2.2.1.8 – Constructs used for retroviral infection

For retroviral infection of FDCP-1 cells a modified pMSCVneo vector, pMSCV-IRES-huCD2 tailless was used (a gift from Dr. Owen Williams). This vector was generated by digesting the parental pMSCVneo with *Eco*R I and *Bam*H I and inserting a linker PCR fragment containing an IRES followed by a human CD2 gene lacking most of its cytoplasmic tail. This allows for detection of infected cells by expression of human CD2 on the surface of infected cells. HA-Mcl-1, HA-127-Mcl-1, HA-Δ127-Mcl-1, HA-Δ157-Mcl-1 and HA-DblMut-Mcl-1 were all cloned by digesting the appropriate pcDNA3.1-HA constructs with *Xba*I, blunt ending these inserts, and then cloning them into a blunt ended *Eco*RI site of pMSCV-IRES-huCD2.

2.2.1.9 – DNA Sequencing

All cloned cDNAs were sequenced after cloning to ensure no detrimental mutations had been introduced to the cDNA during cloning. To 6 μl ReadyMix reaction mix (Perkin Elmer) was added 1 μl (3.2 pmol/μl) of an appropriate sequencing primer and 300 – 500 ng plasmid DNA. The reaction volume was adjusted to 15 μl with sterile water. Conditions used for PCR were: 25 cycles of 96°C for 30 sec, 50°C for 30 sec, 60°C for 4 min. Reactions were cleaned up by precipitating with 3 μl of 3 M sodium acetate pH 5.5 in 50 μl of 100% ethanol for at least 1 hour at -20°C. This was followed by two washes with 70% ethanol. Pellets were then dried and run on an ABI PRISM 377 DNA Sequencer (Perkin Elmer). Sequencing was analysed using EditView™ Automated DNA Sequence Viewer version 1.0.1 (Perkin Elmer) and MacVector version 6.5 (Oxford Molecular Ltd.).

2.2.1.10 – Preparation of competent cells

A single colony of *E. coli* was picked into 5 ml of psi broth and grown overnight at 37°C. This was then added to 100 ml of psi broth prewarmed to 37°C and incubated at 37°C with vigorous shaking until $OD_{550} \approx 0.48$. The culture was then transferred to pre-chilled Falcon tubes and allowed to cool on ice for 10 min. Cells were pelleted by centrifugation at 4°C and 3,000 × *g* for 10 min. Pellets were very gently resuspended in 30 ml ice-cold TFBI and kept on ice for 20 min. Cells were again pelleted as before and then gently resuspended in 4 ml ice-cold TFBII. Competent bacteria were dispensed into 100 µl aliquots in 1.5 ml microfuge tubes, snap-frozen in an ethanol/dry-ice bath and stored at -80°C.

2.2.1.11 – Transformation of competent bacteria

50 ng of DNA or 2.5 µl of ligation mix were placed in a pre-chilled 14 ml Falcon tube. This was placed on ice and 50 µl of competent cells added and incubated on ice for 30 min. Cells were incubated at 42°C for 90 sec and immediately placed on ice for 2 min. 400 µl of LB broth was added to the transformation mix and this was then incubated at 37°C for 45 min. A 200 µl aliquot of this culture was then plated out on LB agar plates supplemented with appropriate antibiotic.

2.2.1.12 – Small scale DNA preparation

This was carried out using a Qiagen mini-prep kit available from Qiagen, following instructions as supplied by the manufacturer.

2.2.1.13 – Large scale DNA preparation

This was carried out using a Qiagen HiSpeed Maxi kit available from Qiagen, following instructions as supplied by the manufacturer.

2.2.2 – Protein

2.2.2.1 - SDS-PAGE analysis

Resolving gels contained 10 – 12% acrylamide (Protogel, National Diagnostics) in 375 mM Tris-HCl pH 8.8 and 0.1% (w/v) SDS. Stacking gels were poured on top of resolving gels and contained 4% (w/v) acrylamide in 125 mM Tris-HCl pH 6.8. Gels were polymerised using 0.1% (w/v) ammonuim persulphate and 0.1% (v/v) TEMED. Cells were harvested at 10^7 cells/ml of lysis buffer and an equal volume of 2× sample buffer added. Samples were boiled at 100°C for 5 min and subsequently kept on ice. For each sample 20 µl lysate, equivalent to 10^5 cells, was loaded per lane. Gels were run at a constant voltage of 85 V until just before the dye ran off the gel.

2.2.2.2 – Coomassie staining of SDS-PAGE gels

Coomassie brilliant blue (250 mg/l) was dissolved in an aqueous solution containing 40% (v/v) methanol and 10% (v/v) acetic acid. SDS-PAGE gels were stained for 2 – 4 hours before destaining with an aqueous solution containing 10% (v/v) methanol and 5% acetic acid (v/v). Gels were then vacuum dried onto 3MM paper (Whatman).

2.2.2.3 – Protein transfer to nitrocellulose membrane

SDS-PAGE gels were incubated in transfer buffer for 5 min and proteins transferred to Hybond-C nitrocellulose membrane (Amersham) using a BioRad Mini Trans-Blot Electrophoretic Transfer system according to the manufacturers instructions. Gels were transferred for 45 min – 1 hour at a constant voltage of 85 V.

2.2.2.4 – Western blotting

Nitrocellulose membranes containing transferred protein were blocked for 2 hours at room temperature in TBS-T containing 5% (w/v) non-fat milk powder (Marvel). Blots were incubated with the appropriate primary antibody, diluted in 5% (w/v) non-fat milk powder in TBS-T, for 2 hours at room temperature or overnight at 4°C. Membranes were then washed 3 times (10 min/wash) in TBS-T and then incubated with horse-radish peroxidase (HRP) conjugated secondary antibody diluted 1/1000 in 5% (w/v) non-fat milk powder in TBS-T. Membranes were again washed 3 times (10 min/wash) in TBS-T and then incubated for 1 min with Enhanced

ChemiLuminescence (ECL) reagent (Amersham). Blots were wrapped in saranwrap and exposed to autoradiography film for between 1 second and 2 hours to detect protein bands.

2.2.2.5 – *In vitro* translation and radioactive labelling of proteins

In vitro translated proteins were generated using the TNT® T7 Quick Coupled Transcription/Translation System from Promega, according to the manufacturers instructions. The pcDNA3.1-HA constructs were used as a template as the vector contains a T7 transcriptional start site up-stream of the initiation codon. Proteins were radioactively labeled by translating in the presence of [^{35}S]-methionine (Amersham).

2.2.2.6 – GST-fusion protein production

BL21 *E. coli* cells containing the appropriate plasmid were grown overnight in LB broth supplemented with 100 μg/ml ampicillin. Cultures were then diluted 1/100 and grown to log phase (OD_{600} = 0.5). Expression of the GST-fusion protein was then induced by the addition of isopropyl β-D-thiogalactoside (IPTG) (Insight Biotechnology). Induced cultures were grown for 2 – 3 hours at 32°C. Bacterial pellets were then pelleted by centrifugation at 2000 × *g* for 10 min. Pellets were washed once in ice-cold PBS. Washed pellets were then resuspended in PBS and cells lysed by sonication. A solution of 10% (v/v) Triton X-100 was added to a final concentration of 1% and then mixed. The lysate was centrifuged at 10,000 × *g* for 5 min to remove insoluble material and intact cells. The supernatant was added to 1 ml of a 50% slurry of glutathione-sepharose beads (Amersham) and mixed gently at room temperature for 5 min. Beads were washed by adding 50 ml of ice-cold PBS, mixing and centrifuging for 1 min at 500 × *g*. This wash was repeated twice and the beads resuspended in 1 ml of PBS, transferred to a 1.5-ml microcentrifuge tube and stored at 4°C. Protein production was analysed by SDS-PAGE gel electrophoresis (Section 2.2.2.1) followed by Coomassie staining (Section 2.2.2.2).

2.2.2.7 – GST fusion protein-affinity purification assay

In vitro translated prey proteins radiolabelled with [^{35}S]-methionine were prepared as outlined in Section 2.1.2.5. 1 µl of the recombinant protein was added to 200 µl of bead binding buffer and spun in a microfuge at maximum speed for 15 min at 4°C. The supernatant was transferred to a clean tube and incubated on ice. 200 µl bead binding buffer was added to 20 µl of GST or GST-fusion protein bound to glutathionine sepharose beads. The sepharose beads mix was added to the recombinant prey protein and incubated for 2 hours with rotation at 4°C. Beads were pelleted by centrifugation at 200 × *g* for 1 min and the supernatant discarded. Pellets were washed 5 times with ice-cold bead binding buffer. Finally beads were resuspended in 25 µl of 1× sample buffer and analysed by SDS-PAGE gel electrophoresis (Section 2.2.2.1). Gels were fixed and stained by Coomassie staining (Section 2.2.2.2) to ensure equal loading of GST and GST-fusion. Gels were then vacuum dried onto 3MM paper and exposed to a phosphorimager screen.

2.2.3 – Yeast two-hybrid protocols

2.2.3.1 – Small scale transformation of Y190

Y190 yeast was streaked out on a YPD plate containing extra adenine. A single clone was picked from this and cultured in 5 ml YPD overnight at 30°C. 20 ml of fresh media was then inoculated with 0.5 ml of the overnight culture and again incubated overnight. The overnight culture was then diluted in fresh media such that the OD_{600} of the culture was 0.15. Culture was grown for another 4 hours until the OD_{600} was 0.6. The cells were then pelleted by centrifugation at 3,000 × *g* for 10 min and the media removed. To generate competent yeast pellets were washed once in ice-cold sterile water and then washed twice in sterile 1 × lithium acetate/TE. After the final wash the pellet was resuspended in 0.5 ml of 1 × lithium acetate/TE and kept on ice at all times. 100 µg of denatured salmon sperm DNA (Clontech) was added to 0.5 µg of plasmid DNA, to a volume of 50 µl, and mixed. To this DNA mix was added 100 µl of competent yeast cells. Following this, 600 µl of lithium acetate/TE/PEG was added and the mixture briefly vortexed. This was then incubated at 30°C for 30

minutes followed by the addition of 70 µl of DMSO. The cells were then mixed gently by inversion and given a heat shock at 42°C for 15 minutes. Cells were cooled on ice for 2 minutes and then the spun briefly (10 seconds at maximum speed in microfuge) to pellet cells. The pellets were resuspended in 100 µl of TE and plated onto appropriate minimal media plates. These were incubated at 30°C for 2 – 3 days until colonies appeared.

2.2.3.2 – Library transformation of Yeast

A single colony of Y190-tBid was picked from a fresh streak-plate. This was used to inoculate 10 ml of minimal broth lacking tryptophan and the culture grown overnight at 30°C. This culture was used to inoculate 50 ml of fresh minimal media lacking tryptophan and again incubated overnight at 30°C. This was then diluted into ~450 ml of fresh and complete media such that the OD_{600} was 0.25. The culture was grown for a further 4 hours until the OD_{600} reached 1.0. The culture was then centrifuged at 3000 × *g* for 10 min to pellet cells and the media removed. The pellets were washed twice with 10 volumes of ice-cold sterile water followed by two washes with 10 volumes of ice-cold lithium acetate/TE. Competent cells were resuspended in a total volume of 4 ml and kept on ice at all times. The DNA mix was prepared by adding 4 mg of denatured salmon sperm DNA (Clontech) to 100 µg of library DNA. To this was added 75 µl of 10 × Lithium Acetate and 10 × TE and the total volume made up to 750 µl with sterile water. The DNA mix was then added to the competent cells and mixed gently by inversion. Next, 24 ml of lithium acetate/TE/PEG was added and mixed gently. The transformation mix was incubated at 30°C for 30 minutes with slow shaking at 60 rpm. Cells were then heat shocked at 42°C for 20 minutes and cooled on ice for a further 2 minutes. The cells were then pelleted by centrifugation at 3000 × *g* for 10 minutes the supernatant removed and the pellet resuspended to a total volume of 10 ml with sterile water. Transformation efficiency was measured by taking 1 µl and 5 µl aliquots and plating onto minimal media plates lacking tryptophan and leucine. The rest of the library transformation was plated out onto large Nunc plates containing minimal media lacking histidine, tryptophan and leucine and containing 17 mM 3-AT. Plates were wrapped loosely to prevent drying and incubated for up to 15 days at 30°C.

2.2.3.3 – Titration with 3-amino-1,2,4-triazole (3-AT)

As the *HIS3* reporter gene is slightly leaky in Y190, incorporation of 3-AT, a specific inhibitor of the gene product, is included in the media to inhibit the leaky HIS3. Thus only when the reporter is fully switched on can the yeast grow and this helps reduce the number of false positives from the yeast two-hybrid screen. A titration of 3-AT is required to identify the best concentration of the compound to use. Appropriate dropout media was prepared and petri dishes containing 0 mM, 5 mM, 10 mM, 15 mM, 20 mM, 25 mM, 30 mM, 40 mM, 50 mM or 60 mM 3-AT were poured. A Y190-tBid colony was picked from a fresh streak and grown overnight in 5 ml dropout broth lacking both tryptophan and uracil. This was diluted 1/40 and 200 µl of this culture was spread on each plate. Plates were incubated for 7 days at 30°C. After this colony growth was observed and colonies were counted to determine the optimum concentration of 3-AT to use. Filter lift assays (Section 2.1.3.3) were also carried out to check for autoactivation of the bait construct.

2.2.3.4 – Filter lift assay

3MM filter paper (Whatman) was cut to size and orientated with respect to the plate to be lifted. The filter paper was placed on the surface of the plate, pressed down firmly and removed swiftly. This was then used to replica plate colonies onto a similar plate with fresh media. Filters were frozen in liquid nitrogen for 5 seconds, removed and allowed to thaw. Following a second freeze thaw step filters were incubated in Z-buffer (20 ml for large plates, and 2 ml for petri dishes) for up to 8 hours at room temperature to allow the blue colour to develop. These filters were used to identify corresponding positive colonies on the replica plates.

2.2.3.5 – PCR from whole Yeast

Prey inserts from the yeast two-hybrid assay were initially amplified directly from yeast by PCR. A stock mix was prepared containing 1× Sigma RED*Taq* buffer, 200 µM dNTPs, 100 ng of both GADF1 (5′-ATGAAGATACCCCACCAAACCC-3′) and GADR1 (5′-TGCACAGTTGAAGTGAACTTGC-3′) primers, 0.05 U/µl of

JumpStart RED*Taq* DNA polymerase in sterile water. Aliquots of 50 µl were placed in 0.2 ml PCR tubes. Using a p200 pipette tip a tiny amount of a yeast colony was picked and smeared to the inside of the PCR tube. Tubes were vortexed to mix and then spun briefly. Conditions used for PCR were: 1 cycle of 94°C for 15 min to break open the yeast; 30 cycles of 94°C 1 min, 56°C for 1 min, 72°C for 5 min; 1 cycle of 72°C for 5 min. PCR products were analyzed on a 1.0% agarose gel and were cleaned up using a Qiagen PCR clean-up kit according to the manufacturer's instructions and eluted in 30 µl of elution buffer.

2.2.3.6 – Sequencing of PCR products

Sequencing PCR products from yeast was carried out in as described in Section 2.1.1.9 using 8 µl of cleaned-up PCR product as a template. Reactions were cleaned up and analysed as above.

2.2.3.7 - Extraction of library plasmid from yeast

Positive clones identified in the yeast two-hybrid assay were picked and grown overnight at 30°C in 5 ml of dropout broth lacking tryptophan and leucine. An aliquot of 1.5 ml was taken from this culture and centrifuged at 13,000 × *g* for 5 min and the supernatant removed. The pellet was then resuspended in 200 µl of Zymolase buffer and incubated at 37°C for 2 hours. Lysis of the cells was then carried out using a Qiagen miniprep kit, according to the manufacturers instructions, and eluted in 30 µl of elution buffer.

2.2.3.8 – Generation of yeast protein extracts

A single colony of yeast was picked into 5 ml of appropriate drop-out media and grown at 30°C overnight. Cultures were diluted 1/100 in 100 ml of fresh media until they had reached a density of OD_{600} 0.4 – 0.6. Cells were pelleted by centrifugation at 3,000 × *g* for 10 min. Pellets were then washed twice with ice-cold sterile water to remove all media. All samples were kept on ice from this point on. The pellet was resuspended in one volume of 2× sample buffer and the made up to five volumes with 3 volumes of 1× sample buffer. To this was added 100 µl of acid washed glass beads

and the samples then vortexed for 5 min in the cold-room. Samples were then boiled at 100°C for 10 min, cooled on ice for 2 min, and then spun at top speed in a microcentrifuge for 10 min. The supernatant was removed to a fresh tube and 15 µl analysed by SDS-PAGE gel electrophoresis (Section 2.1.2.1) and western blotting (Section 2.1.2.4). The remainder was stored at -70°C.

2.2.4 – Cell biology

2.2.4.1 – Cell lines and culture

Jurkat T-cells were grown in RPMI 1640 and supplemented with 10% FCS, 2 mM glutamine, 0.5 U/ml penicillin and 0.5 µg/ml streptomycin. FDCP-1 cells (ATCC) were grown in RPMI 1640 and supplemented with 10% FCS, 2 mM glutamine, 0.5 U/ml penicillin, 0.5 µg/ml streptomycin, 1 mM HEPES, 0.0004% β-mercaptoethanol and 10% IL-3 conditioned media from WEHI-3B cells. The 293 derived LinXE virus packaging cell line was maintained in DMEM supplemented with 10 % FCS, 2 mM glutamine, 0.5 U/ml penicillin, 0.5 µg/ml streptomycin and 75 µg/ml hygromycin (Calbiochem). All cell lines were maintained at 37°C with 5% CO_2 unless otherwise stated.

2.2.4.2 – Induction of apoptosis in Jurkat T cells

For apoptosis assays, cells were seeded at 1 - 2×10^6 cells/ml and treated with 300 ng/ml CH11 anti-Fas (Upstate Biotechnology), 25 µM etoposide or 1 µM staurosporine for up to 6 hours. After treatment, cells were harvested and washed with ice-cold PBS. One-fifth of the cells were analysed by flow cytometry by staining with Annexin V-PE (BD Pharmingen) and 7AAD to measure apoptosis. The other four-fifths were pelleted and lysed in sample buffer for analysis by western blot.

2.2.4.3 – Analysis of apoptotic cells by flow cytometry

Analysis of cells was measured by staining cells with 7AAD and with Annexin V-PE. Cells were resuspended in Annexin staining buffer which contains high calcium

levels. Annexin V-PE was added at a dilution of 1:200. Samples were incubated at room temperature for 10 min. 7AAD was then added to a final concentration of 10 μg/ml. Samples were then analysed on a Beckman Coulter flow cytometer. For analysis of cells that had been infected with constructs containing an IRES-huCD2, cells were first stained with anti-huCD2-FITC for 5 min at a dilution of 1:200.

2.2.4.4 – Preparation of Apoptotic lysate and *in vitro* assay for Mcl-1 cleavage

Jurkat T cells were seeded at 1×10^6 cells/ml and treated for 4 hours with or without CH11 anti-Fas (Upstate Biotechnology). Cell lysates were prepared as described in Sun et al. (1999). Briefly, 2×10^7 cells were pelleted and washed in ice-cold PBS. Cells were then resuspended in PIPES buffer to a volume of 166 μl/10^8 cells. This was followed by three cycles of freezing in liquid nitrogen followed by thawing rapidly at 37°C. Samples were then spun in benchtop centrifuge for 30 mins at 16,000 × *g*. Supernatant was transferred to a fresh tube and spun at 100,000 × *g* for 45 mins. The S100 fraction containing active caspases was collected and the protein concentration determined using Bio-Rad protein assay according to the manufacturers instructions. Aliquots were stored at - 80°C. Human and mouse HA-Mcl-1 were *in vitro* translated as per Section 2.1.2.5. Cell lysate was added to PIPES buffer to a final volume of 48 μl. For assays using recombinant active caspase-3 (BD Biosciences), active caspase-3 was added to a final volume of 48 μl with buffer used according to the manufacturers instructions. Caspase inhibitor zVAD-fmk (Enzyme System Products) or DMSO as a control was then added and incubated at 37°C for 15 min. Finally 1.0 μl of the radiolabelled translated protein was added to the lysate, mixed by pippetting and incubated at 37°C for 2 hours. The reaction was terminated by the addition of 50 μl of 2× sample buffer. 20 μl of this was then resolved on a 12 % SDS-PAGE gel (Section 2.1.2.1). Following this the gel was incubated in fixing solution for 15 min, vacuum dried onto 3MM filter paper and exposed to a phosphorimager screen overnight.

2.2.4.5 – Transfection of Jurkat T cells

The day before transfection Jurkat cells were seeded at 2×10^5 cells/ml. The following day cells were washed in OptiMEM and constructs were co-transfected with GFP using Lipofectamine PLUS (Invitrogen) according to the manufacturers instructions. Generally 1.5 μg of DNA was transfected (Mcl-1 construct:GFP;9:1) per well of a six well plate. 24 hours after transfection cells were treated with either 300 ng/ml CH11 anti-Fas or 25 μM etoposide for up to 6 hours as in Section 2.1.4.2 above. After treatment, cells were harvested and washed with ice-cold PBS. Cells were analysed by flow cytometry by staining with Annexin V-PE (BD Pharmingen) and 7AAD to measure apoptosis. For analysis, only GFP positive cells were examined, and of these only those that were both GFP positive and Annexin V positive were taken to be transfected and apoptotic.

2.2.4.6 – Infection of FDCP-1 cells

Constructs for infection were first transfected into the LinXE packaging cell line using Lipofectamine (Invitrogen) according to the manufacturers instructions. Generally for each construct a 10 cm plate was transfected with 8 μg DNA. 24 hours after infection the media was replaced. Following incubation for an additional 24 hours 10 ml viral supernatant was collected for each construct. FDCP-1 cells for infection counted and 1×10^6 cells resuspended in 2 ml viral supernatant containing 8.0 μg/ml polybrene to give a concentration of 1×10^5 cells/ml. Cells were spun at 1800 rpm for 45 mins at 32°C and then incubated at 32°C with 5% CO_2 for 2 hours. Most of the supernatant was carefully removed and a further 2 ml viral supernatant containing polybrene added. The cells were spun as before and incubated at 32°C with 5% CO_2 for a further 4 hours. The supernatant was again carefully removed and replaced with complete media and the cells incubated as normal overnight. The following morning media was removed from the cells and 4 ml viral supernatant containing polybrene was added and spun as before. Cells were incubated for 6 hours at 32°C with 5% CO_2. Following this the media was replaced with complete media and the cells incubated as normal. The infected populations were expanded and infection efficiency was measured by flow cytometry using an anti-CD2-FITC

antibody (clone RPA-2.10, eBioscience). Generally infection efficiency of 80% upwards was observed.

2.2.4.7 – Cycloheximide treatment of FDCP-1 cells

FDCP-1 cells were split to 2×10^5 cells/ml the day before treatment. On the day of treatment 2 ml of cells at 5×10^5 cells/ml were placed in wells of a 6-well plate. Cells were then treated for 0, 10, 20, 30, 60, 90, or 120 min with 30 μg/ml cycloheximide (CHX), harvested and washed with ice-cold PBS before being lysed in Laemmli buffer. Samples were analysed by Western blotting and probed with anti-Mcl-1 or anti-HA antibodies. Blots were also probed with anti-α-tubulin to check for loading.

2.2.4.8 – Growth Factor Withdrawal assays

FDCP-1 cells were prepared for growth factor withdrawal assays by first washing cells three times in RPMI-1640. Cells were then resuspended in media containing RPMI-1640 with 1% FCS, 2 mM glutamine, 0.5 U/ml penicillin, 0.5 μg/ml streptomycin, 1 mM HEPES, and 0.0004% β-mercaptoethanol and maintained at 37°C with 5% CO_2. Apoptosis was measured by harvesting cells, resuspending in hypotonic buffer and addition of propidium iodide to examine DNA content of the cells. The percentage of sub-G1 cells was used as a measure of apoptosis.

3 - Yeast Two-Hybrid

3.1 – Introduction

3.1.1 – BH3 only proteins and apoptosis

As outlined in Chapter 1, the Bcl-2 family of proteins are important regulators of apoptosis. Within this family the BH3 only proteins play an important role in responding to stimuli by sensitising (e.g. through Bad) or activating (e.g. through Bid and Bim) cells to apoptosis (Letai et al., 2002). We are interested in the "BH3 only" protein Bid. While its cleavage by caspase-8 is well characterised and it is well known that the truncated form of Bid (i.e. tBid) is the active form of the protein (Li et al., 1998; Luo et al., 1998) it is not clear how tBid is targeted to the mitochondria and how it regulates apoptosis in this setting.

We decided to use tBid as a bait protein in a yeast two-hybrid screen to identify novel interaction partners for the protein and to further clarify how this protein contributes to apoptosis.

3.1.2 – Yeast two-hybrid

Protein-protein interactions are important for all biological systems. The yeast two-hybrid assay is a technique that was developed to examine and identify protein-protein interactions (Fields and Song, 1989). Yeast two-hybrid technology has proven to be particularly useful for analysis of proteins involved in apoptosis. A number of the Bcl-2 family of proteins were first identified using the yeast two-hybrid assay (Han et al., 1996; Yang et al., 1995), and much of the work characterising interaction between family members as well as the domains important for these interactions has been done using this technique (Sato et al., 1994; Sedlak et al., 1995; Wang et al., 1998). In order to further our understanding of the role of tBid in apoptosis, we have used tBid in this assay to screen a library for novel interaction

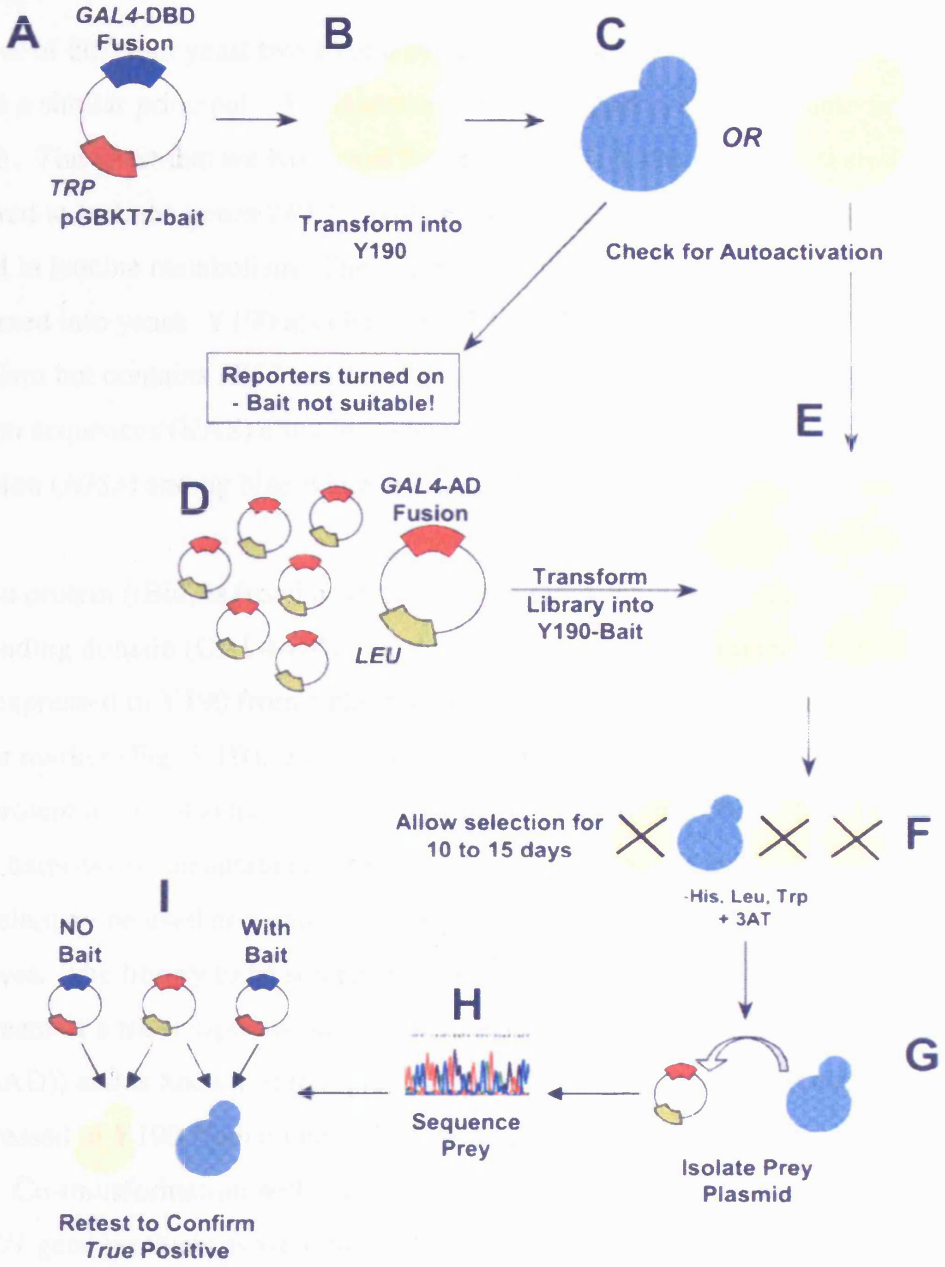


Figure 3-1: Overview of the yeast two-hybrid system. This is explained in the text below

partners. The principle behind this technology is reviewed in (Fields and Sternglanz, 1994) and is summarised in Figure 3-1.

A number of different yeast two-hybrid systems have been established and all are based on a similar principal. We have used a GAL4 based system available from Clontech. The yeast that we have used for our screen is Y190. This yeast strain is engineered to lack the genes *TRP1*, involved in tryptophan metabolism and *LEU1* involved in leucine metabolism. These genes are used to select for plasmids transformed into yeast. Y190 also lacks a normal *HIS3* gene involved in histidine metabolism but contains *HIS3* and *LacZ* genes under the control of GAL4 upstream activation sequences (UAS) allowing selection of interacting proteins by nutrient deprivation (*HIS3*) and by blue/white selection (*LacZ*).

A known protein (tBid) is fused downstream of a DNA binding domain (the GAL4 DNA binding domain (GAL4-DBD)) and is known as the 'bait' protein (Fig. 3-1A). This is expressed in Y190 from a plasmid containing a tryptophan nutritional selection marker (Fig. 3-1B). At this stage it is important to ensure that the bait fusion protein does not activate the reporter genes alone (Fig. 3-1C). If this is the case the bait may be unsuitable for this assay. Alternatively, specific domains within the protein may be used as a bait, providing that they do not activate the reporters themselves. The library to be screened, generally a cDNA library, is fused downstream of a transcriptional activation domain (the GAL4 activation domain (GAL4-AD)) and is known as the 'prey' protein (Fig 3-1D). Our prey fusion-proteins are expressed in Y190 from a plasmid containing a leucine nutritional selection marker. Co-transformation with these plasmids results in the expression of the *TRP1* and *LEU1* gene products as well the GAL4-fusion bait and prey proteins. This allows growth of Y190 on dropout media lacking either tryptophan and/or leucine and potential activation of the reporter *HIS3* and *LacZ* genes.

The GAL4-DBD-bait vector is transformed into yeast (Fig. 3-1B), where the fusion protein is expressed and localises to the nucleus. Here it binds to the *GAL4* UAS that controls reporter gene expression. The bait fusion protein should not be able to active transcription of the reporter genes itself. In Y190 the *HIS3* reporter gene is slightly

leaky, therefore a specific inhibitor of the *HIS3* gene product, 3-amino-1,2,4-triazole (3-AT), is incorporated into the media to inhibit this low level of *HIS3* expression. Prior to screening, the amount of 3-AT incorporated into the media is optimised. Once these conditions have been established the yeast containing bait is transformed with a GAL4-AD-prey vector containing the library (Fig. 3-1D). This fusion protein is also localised to the nucleus and here the bait and prey can interact with each other. Interaction of the bait and prey bring the GAL4-DBD and –AD into close contact allowing transcription of the reporter genes. After transformation with a library, the yeast are plated out on a minimal dropout media and allowed to grow for up to 15 days (Fig. 3-1F). Yeast containing a prey vector expressing a fusion protein that does not interact with the bait should not grow, while prey fusion proteins interacting with the bait protein switch on the reporters and grow successfully. Filter lift assays are used to check for *LacZ* expression on the colonies that grow successfully. The colonies that are blue are re-streaked and the prey vector isolated (Fig 3-1G). The library insert is sequenced to identify the gene and to confirm that it is in the correct open reading frame (ORF) downstream of the *GAL4*-AD in order to express a functional fusion gene (Fig 3-1H). This will refine the number of positives, as some of the constructs will not be in the correct ORF with the upstream *GAL4*-AD. To confirm positive and *true* interactions, the isolated prey constructs are co-transformed into yeast with a vector containing a bait fusion protein or lacking the bait fusion, grown on minimal media and filter lift assays carried out again to show the interaction is reproducible and that the prey fusion protein is not able to switch on the reporters without the bait being present (Fig 3-1I).

Once *true* positive interactions partners for the bait protein have been identified, these need to be confirmed outside of the yeast system. We have confirmed these interactions using a GST-pull down assay.

3.1.3 – GST pull-downs

The GST pull-down assay is an *in vitro* assay that is used to characterise and show the ability of proteins to interact with one another. This assay can be a very sensitive and

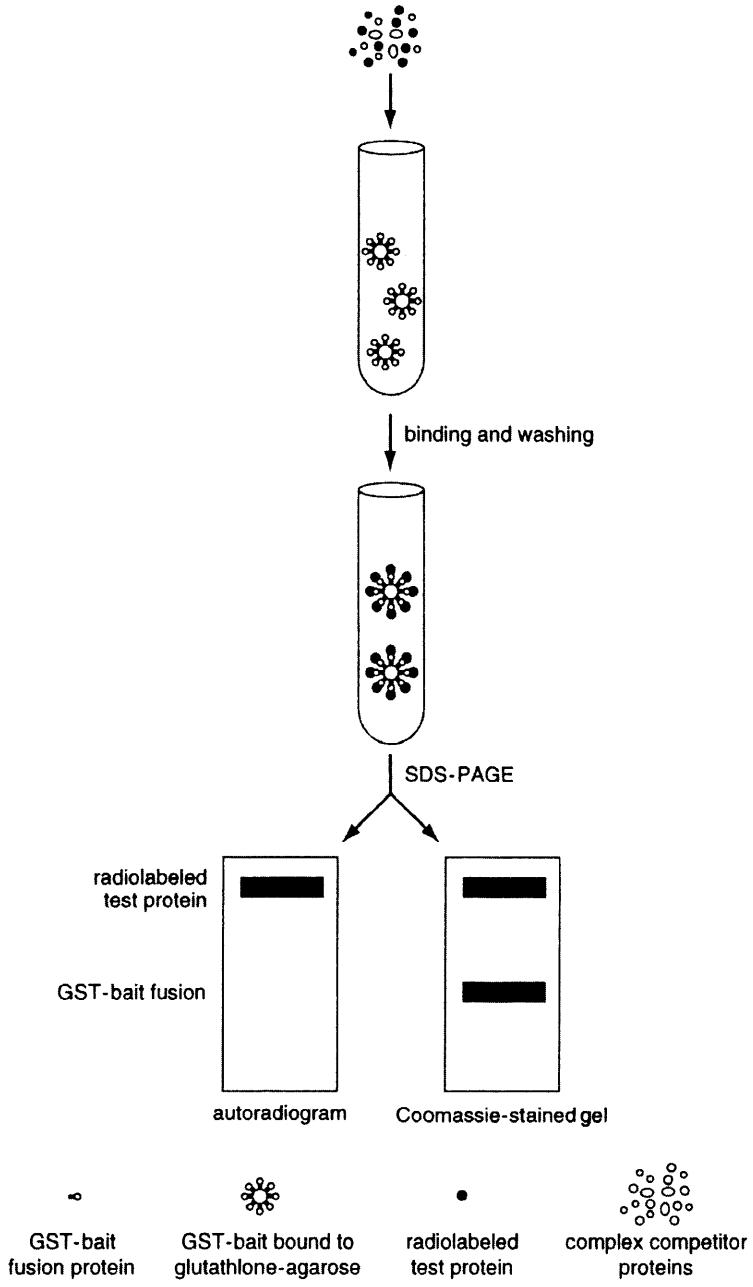


Figure 3-2: Schematic representation of GST pulldown assay. Taken from *Current Protocols in Molecular Biology.*

is often used in conjunction with other assays to independently confirm the ability of two proteins to interact with each other. This system is shown in Figure 3-2. Glutathione-*S*-transferase (GST) is a small protein of about 25 kDa that has a strong affinity for glutathione while having few non-specific interactions with other proteins. Bacterial systems are used to inducibly express GST fusion proteins that can be easily purified from lysed cells under non-denaturing conditions by absorption onto glutathione sepharose or agarose beads.

The system that we have used is a GST-fusion system that is commercially available from Amersham Biosciences. The genes of interest are fused to GST by cloning them downstream of and in frame with GST in a bacterial expression vector. The fusion gene produced is then incubated with a test protein to check for interaction. Using an *in vitro* translated bait protein labelled with [^{35}S]-methionine gives a high degree of sensitivity and allows for accurate quantitation by phosphorimaging or densitometry.

3.2 – Results

3.2.1 – Constructs for the yeast two-hybrid system

Mouse Bid and tBid were cloned by PCR from a mouse Bid cDNA that was a gift to us from Dr. Stanley Korsmeyer (Dana-Faber Cancer Institute, Boston, USA). The PCR products were then cloned into the bait vector pGBKT7 (Clontech) (Fig. 3-3A) in-frame with the upstream GAL4-DBD. The bait construct containing full-length Bid will be referred to as pGBid from here on, and the bait construct containing tBid will be referred to as pGtBid. A number of reports indicate that tBid can directly interact with Bax to induce a conformational change in the protein to result in its activation (Desagher et al., 1999; Eskes et al., 2000). Thus we decided to use Bax as a positive interacting prey for tBid in our yeast two-hybrid assay.

Mouse Bax was cloned by PCR from a mouse *baxα* cDNA construct that we have in our lab. This PCR product lacks the last 21 amino acids of Bax, which encodes for its transmembrane domain. The presence of the transmembrane domain may result in inappropriate localisation of the Gal4-AD-Bax fusion protein and its removal ensures its nuclear localisation. The ΔBax construct was cloned into pGADT7 (Clontech) (Fig. 3-3B) in-frame with the upstream GAL4-AD. This construct will referred to as pGABax from this point on.

A number of other vectors were used as positive controls for the yeast two-hybrid assay and are also commercially available from Clontech. These include pCL-1, containing a full open reading frame for the GAL4 protein (Fig. 3-4A); pVA3-1, a bait vector containing murine p53 protein (amino acids 72-390) fused to the GAL4-DBD (Fig. 3-4B); pTD1-1, a prey vector containing the SV40 large T-antigen (amino acids 87-708) fused to the GAL4-AD (Fig. 3-4C), and which interacts very strongly with the p53 region expressed from pVA3-1; and finally pLAM5'-1, which may be used as a false positive detection plasmid as it contains the human lamin C protein (amino acids 66-230) fused to the GAL4-DBD (Fig. 3-4D) and is relatively inert.

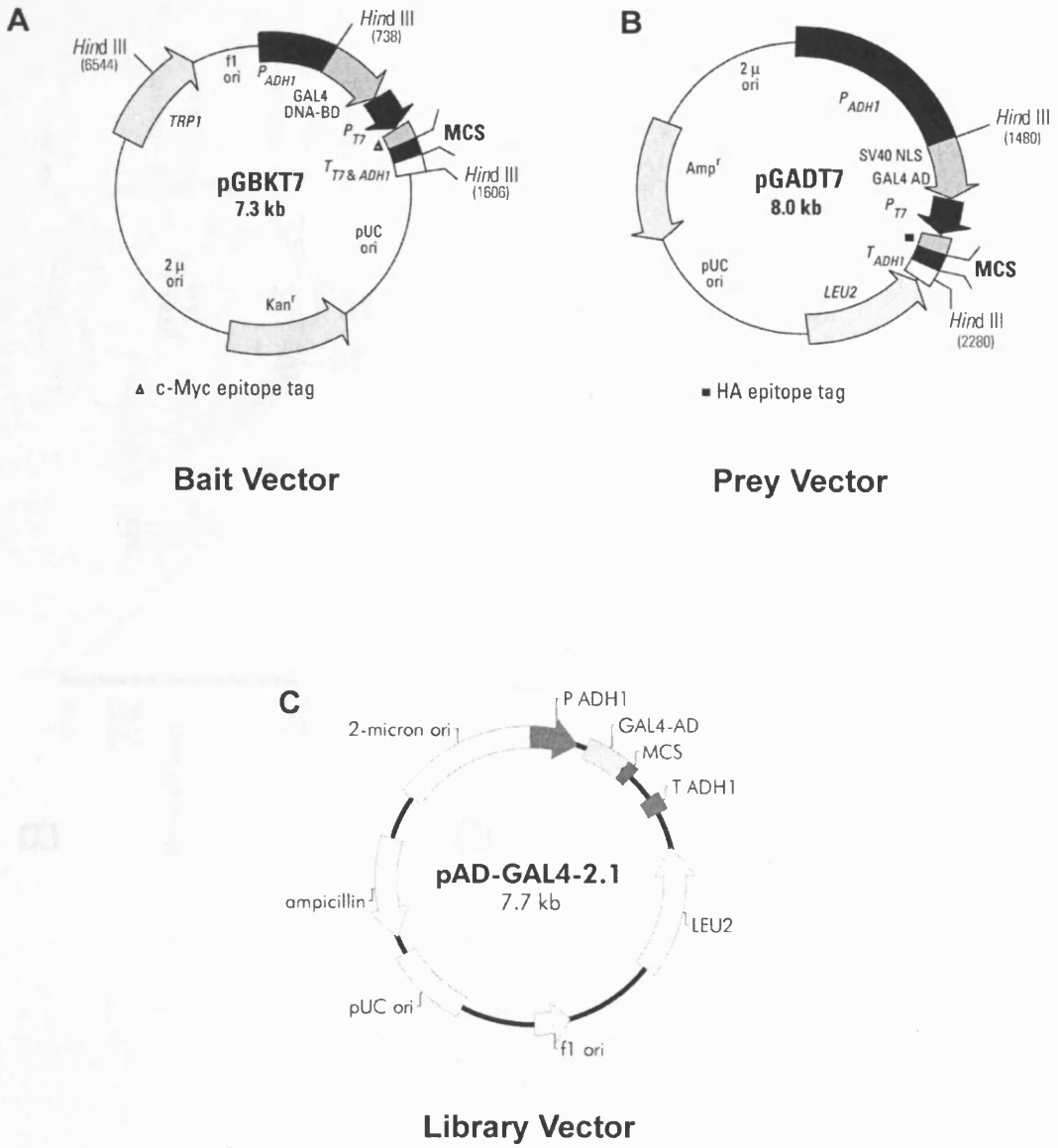


Figure 3-3: Vectors used for the yeast two-hybrid assay. The bait vector pGBKT7 (A) and the prey vector pGADT7 (B) were purchased from Clontech and used to express bait and prey fusion proteins in yeast. The prey vector pAD-GAL4-2.1 (Stratagene) in (C) was used in the cDNA library screened.

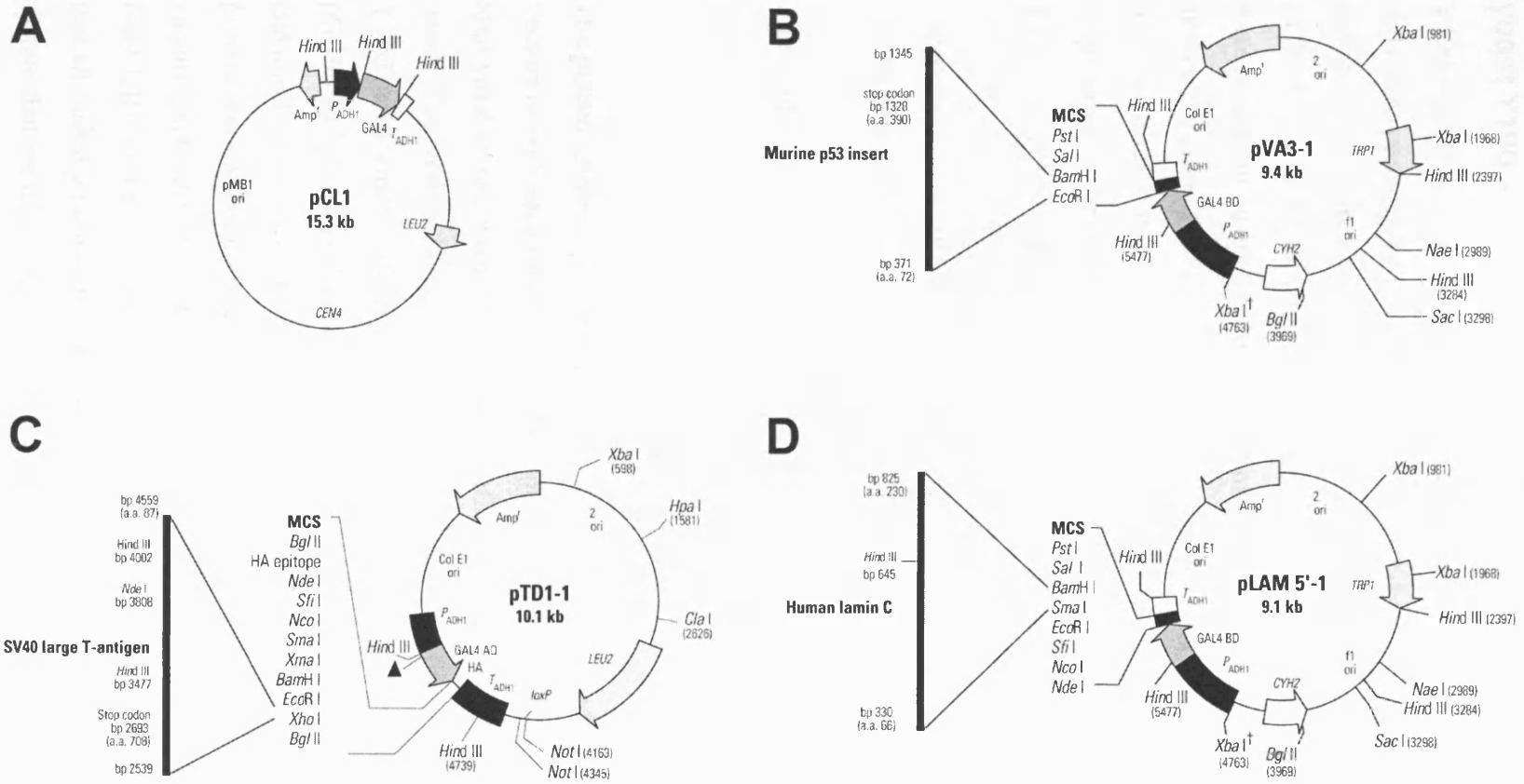


Figure 3-4: Control vectors used in the yeast two-hybrid assay. (A) The pCL1 vector contains a full GAL4 gene and allows expression of reporter constructs in Y190. The pVA3-1 bait vector (B) containing a fragment of murine p53 and the pTD1-1 prey vector (C) containing a fragment of the SV40 large T-antigen produce fusion proteins that interact with each other and activate transcription of reporter genes when co-transformed into yeast. (D) The pLam5'-1 vector contains human lamin C protein and may be used as a control to test for false positive interactions in the two-hybrid assay.

3.2.2 – Expression of / Transformation of the plasmids into the yeast Y190

Y190 yeast was used for all two-hybrid assays during this project. Competent yeast cells were prepared by the lithium acetate method already outlined in Chapter 2.2.3.1. Initial transformations with Y190 used the control plasmids pCL-1, pVA3-1 and pTD1-1. Fig. 3-5A outlines the transformations carried out. These transformations were plated out on appropriate dropout media (lacking tryptophan and leucine) and allowed to grow at 30°C for 2 to 3 days. Filter lift assays were carried out to check for expression of the *LacZ* reporter gene. As expected the pCL-1 plasmid transformed yeast expressed the *LacZ* reporter gene (Fig 3-5B). Co-transforming Y190 with pVA3-1 and an empty prey vector (Fig 3-5C), or pTD1-1 with an empty bait vector (Fig 3-5D) did not induce expression of the *LacZ* reporter. However, co-transformation of pVA3-1 with pTD1-1 resulted in the colonies turning blue when treated with the Z-Buffer (Fig 3-5E).

This confirmed that the Y190 was behaving as expected and that the *LacZ* reporter was working efficiently and did not appear to be leaky. We then tested our bait fusion proteins in the system.

The pGBid, pGtBid, pGABax plasmids were co-transformed with empty bait or prey vectors or with each other into Y190 as outlined in Figure 3-6A. Western blots of total yeast extract were carried out to confirm expression of the fusion proteins within yeast. The western blots in Figure 3-7 each show untransformed Y190 (Lane 1), Y190 transformed with empty vector (Lane 2), and two clones for each of pGBid, pGtBid and pGABax (Lanes 3 and 4). Antibodies raised against full-length murine Bid and Bax were used to detect both Bid and Bax respectively. The tBid fusion protein was undetectable using the antibody against full-length Bid. Instead we used an anti-Gal4 antibody to detect the fusion protein. This antibody also detects the Gal4-DBD in the vector only lane (Lane 2) of the tBid panel. These westerns show that all fusion proteins appear to be expressing correctly within the yeast two-hybrid system that we have used.

A

Transformation (i.e. Filter lift)	Bait	Prey	Blue
B	pCL1		+
C	pVA3-1	Empty vector	-
D	Empty vector	pTD1-1	-
E	pVA3-1	pTD1-1	+

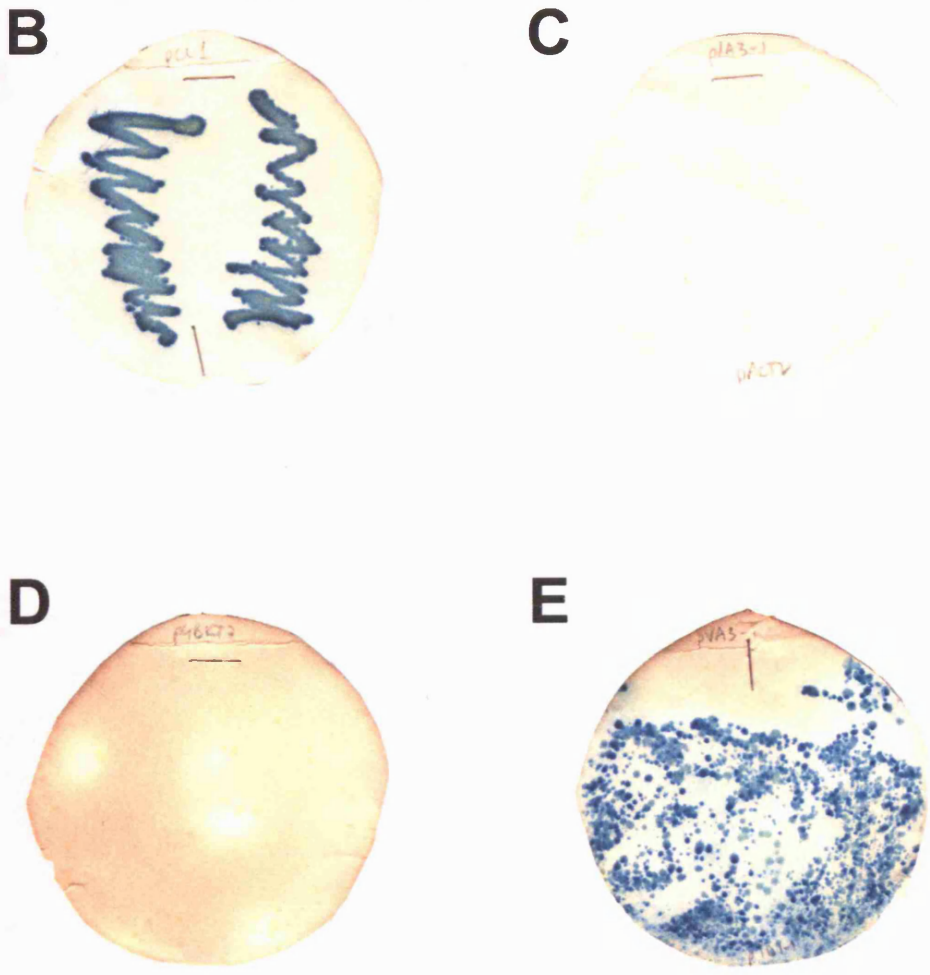


Figure 3-5: Control transformations in Y190. Transformations with the plasmids shown in Figure 3-4 are outlined in (A) above and the corresponding filter lift assays are shown in (B), (C), (D) and (E).

A

Transformation (i.e. Filter lift)	Bait	Prey	Blue
B	pGBid	Empty vector	-
C	pGtBid	Empty vector	-
D	Empty vector	pGABax	-
E	pGBid	pGABax	-
F	pGtBid	pGABax	+

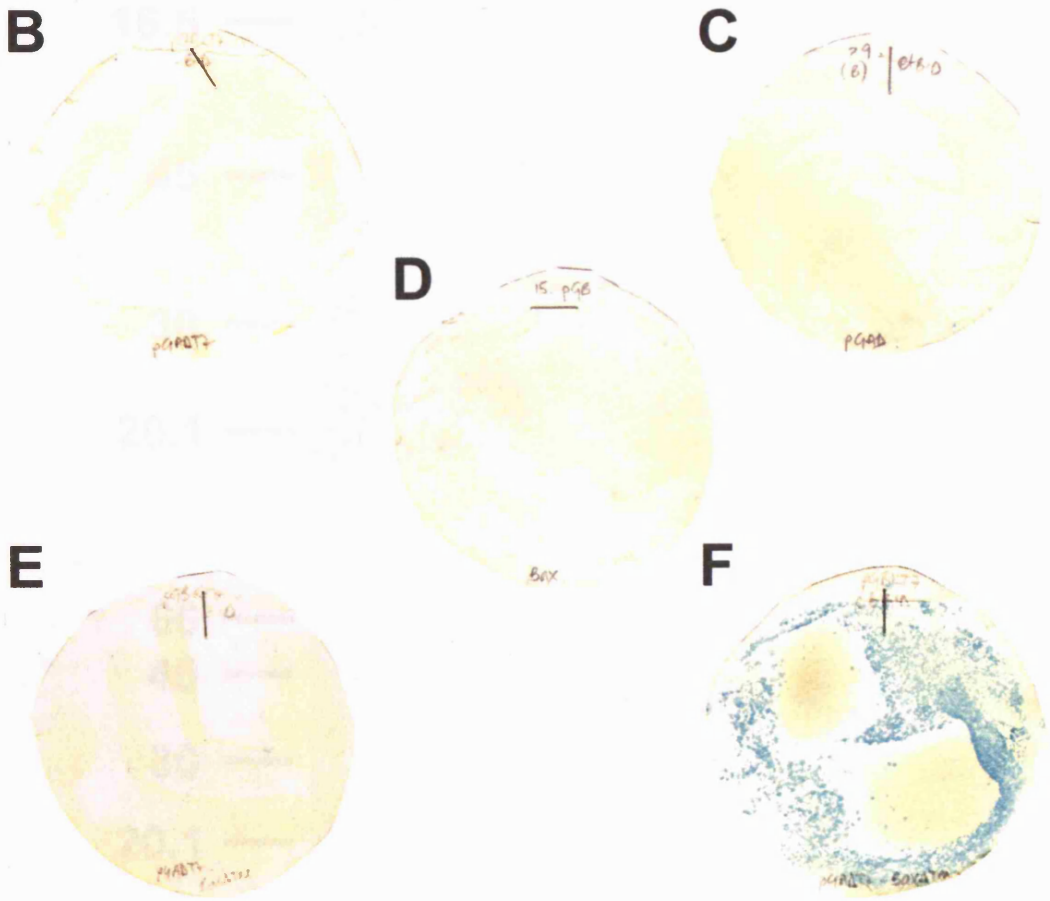


Figure 3-6: Transformation of Y190 with Bid and Bax fusion proteins. Plasmids containing Bid, tBid and Bax fusion proteins were transformed into Y190 as outlined in (A). Filter lift assay results (B – F) show the ability of tBid and Bax to interact and induce expression of the *LacZ* reporter gene in Y190.

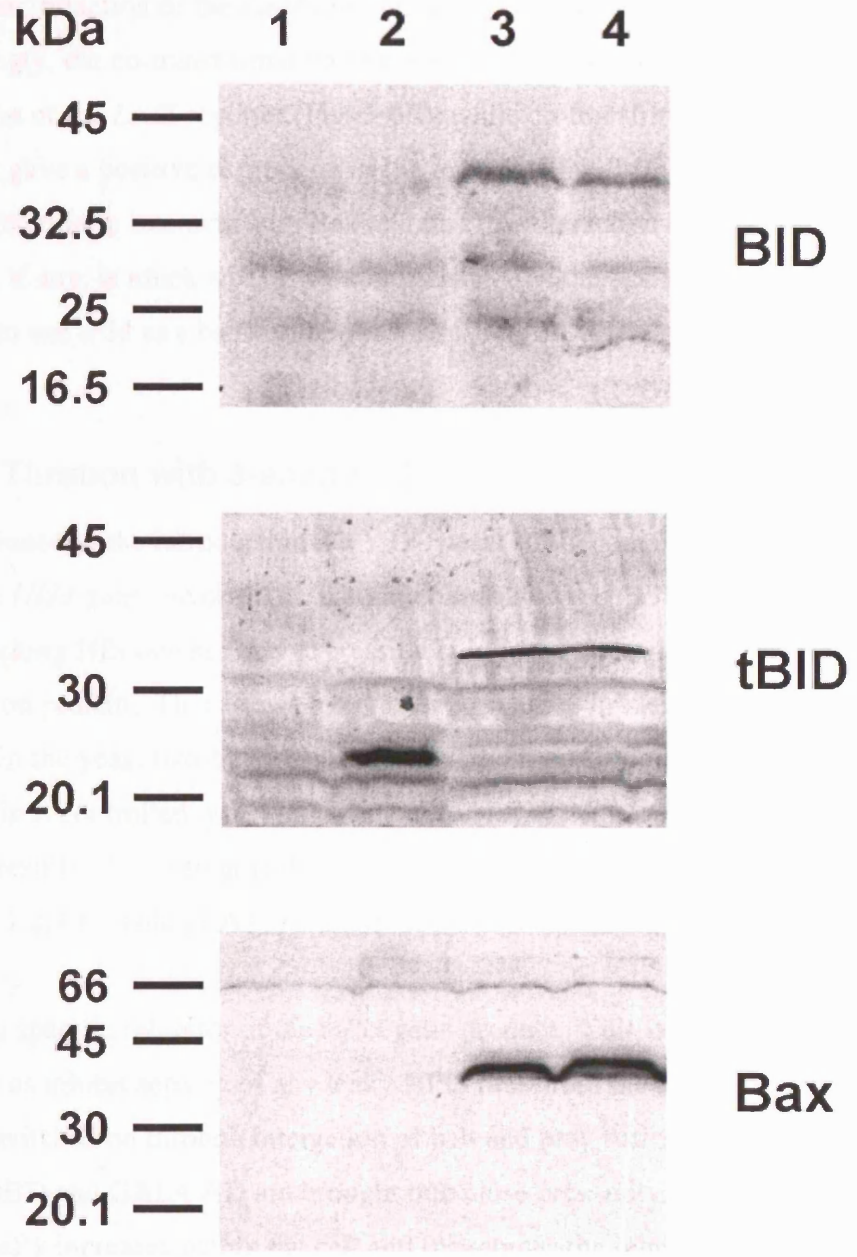


Figure 3-7: Expressed fusion proteins in Y190. Expression of Gal4-DBD-Bid, –tBid and Gal4-AD-Bax in Y190 was detected by western blot. Anitbodies raised against full-length murine Bid and Bax were used to detect full-length Bid and Bax fusion proteins respectively. An anti-Gal-4 antibody was used to detect the tBid fusion protein as the Bid antibody was unable to detect the protein.

Filter lift assays on Y190 transformed with bait or prey fusion constructs alone showed no induction of the *LacZ* reporter as expected (Fig 3-6B, C and D). Interestingly, the co-transformation of pGBid with pGABax was also negative for expression of the *LacZ* reporter (Fig. 3-6E), while co-transformation of pGtBid with pGABax gave a positive expression of the reporter (Fig. 3-6F). This suggests that tBid preferentially interacts with Bax and that the interaction between full length Bid and Bax, if any, is much weaker by comparison. On the basis of these results it was decided to use tBid as a bait for the yeast two-hybrid screen.

3.2.3 – Titration with 3-amino-1,2,4-triazole (3-AT)

As mentioned in the introduction the Y190 yeast strain contains a second reporter gene, the *HIS3* gene, involved in histidine biosynthesis. Y190 is unable to grow on media lacking HIS unless *HIS3* expression is induced through interaction of a bait and prey fusion protein. This allows for greater stringency in identifying *true* interaction partners in the yeast two-hybrid system. However, this reporter is slightly leaky and unless this is controlled, yeast may be able to grow giving in a number of false positive results. To overcome the effect of this leaky expression from the *HIS3* locus 3-amino-1,2,4-triazole (3-AT) is incorporated into the media

3-AT is a specific inhibitor of the *HIS3* gene product. This is added to the dropout media so as inhibit activity of any leaky HIS3 present in the cell. When the *HIS3* gene is switched on through interaction of bait and prey fusion proteins, so as the GAL4-DBD and GAL4-AD are brought into close proximity, the level of HIS3 dramatically increases within the cell and overcomes the inhibitory effect of the 3-AT allowing the yeast to grow normally. Y190 expressing the tBid bait fusion was titrated with increasing amounts of 3-AT to determine the optimum amount to use in order to inhibit growth when the reporter is not switched on.

3-AT concentrations of 0 mM, 5 mM, 10 mM, 20 mM, 25 mM, 35 mM, 40 mM, 45 mM, 50 mM and 60 mM were used in a dropout media lacking tryptophan and histidine. Y190-tBid was grown in liquid culture over night and then plated onto dropout media containing 3-AT. These plates were incubated for 7 days at 30°C. Plates were initially examined for growth of colonies and subsequently filter lift

assays carried out to check for auto-activation of the reporter genes. This also allows us to examine any possible mutagenic effect that the addition of 3-AT may have on reporter gene expression as well as general toxicity to the yeast.

The results of these assays summarised in Table 3.1. Growth of the Y190-tBid yeast on a media containing 3-AT resulted in very little autoactivation of the reporter even at higher concentrations. There were a couple of large colonies that grew on plates at much higher concentrations of 3-AT (i.e. > 30 mM). However, concentrations of 15 to 20 mM 3-AT were sufficient to minimise growth as a result of leaky expression of the *HIS3* gene. Therefore it was decided to use a concentration of 20 mM 3-AT for the tBid yeast two-hybrid screen.

3.2.4 – Apoptotic thymocyte library used for screen

The library used for the yeast two-hybrid screen was a cDNA library prepared from mouse primary thymocytes undergoing apoptosis and was a kind gift from Dr. Gabriel Gil-Gómez (IMIM, Barcelona). Mouse (FVB) thymocytes were irradiated with 5 Gy and cultured for 5 hours. RNA was prepared from these thymocytes and cDNA generated by a random priming method. The cDNA inserts were cloned into the *Eco*RI and *Xba*I sites of pAD-Gal4-2.1 (Stratagene). The library had over 90 % recombinants with an average insert size of 1.5 kb and contained approximately 1.6×10^6 recombinant clones.

3.2.5 – Large scale cDNA library transformation

Y190 containing tBid was streaked on an agar plate containing appropriate dropout media. This was allowed to grow for 2 days at 30°C and then a single colony picked into 5 ml of similar dropout broth. This was grown overnight at 30°C with shaking and used to inoculate 500 ml of YPDA media the following morning such that the OD_{600} was 0.28. This large culture volume was grown at 30°C with shaking and the cells harvested at $OD_{600} = 0.85$. The cells were pelleted and made competent by washing in lithium acetate and were transformed with 240 μg of the cDNA library as

3-AT Conc.	No. of Colonies	Colony Size	Comments	LacZ +ives
0 mM	Confluent Growth	-	No colonies - complete confluent growth	-
5 mM	Confluent Growth	-	No colonies - complete confluent growth	-
10 mM	200 - 300 colonies	~0.5-1.0 mm	These colonies look small - not growing well	-
15 mM	100 - 200 colonies	<0.5 mm	Very small colonies - minimal growth	-
20 mM	~50 colonies	tiny	Very little background growth	-
25 mM	~ 50 colonies	tiny	-	1
30 mM	4-6 large ~20 tiny	~1.0 mm <0.5 mm	-	-
35 mM	6-8 large 10-20 tiny	~1.0 mm <0.5 mm	-	-
40 mM	3 to 4	<0.5 mm	-	-
45 mM	3 to 4	<0.5 mm	-	-
50 mM	2	<0.5 mm	-	-
60 mM	1	<0.5 mm	-	-

Table 3.1: Summary of results for titration of Y190-tBID with 3-amino 1,2,4-triazole

outlined in Chapter 2 (Section 2.2.3.2). After transformation the cell pellet was made up to 10 ml with sterile water and 1 ml was spread on each of 10 large square NUNC plates containing dropout media lacking tryptophan, leucine, histidine and containing 20 mM 3-AT. Transformation efficiency was calculated by plating 1 μl and 5 μl aliquots from the large scale transformation on -Leu, -Trp dropout media. This allows for growth of yeast containing both plasmids. Colonies were counted on each plate 3 days after transformation. The 1 μl plates contained 315 colonies, and the 5 μl plate contained approximately 1700 colonies. This gives a total number of transformants of between 3.1 to 3.4×10^6 (and a transformation efficiency of 1.3 to 1.4×10^4 cfu/μg DNA). Given that the total number of recombinants in the library is estimated to be 1×10^6 every cDNA should be screened against tBid.

Library plates were incubated for 17 days at 30°C to allow recovery and growth of yeast. After incubation each plate had 5 to 10 large colonies of up to 10 mm in diameter. These had started to turn slightly pink, indicating a lack of adenine. There were also at least 10 to 20 colonies of ~ 5 – 10 mm in diameter, these were still quite pale. Finally there were lots of colonies about 1 – 2 mm diameter whose growth had been inhibited by the presence of 3-AT. Replica plates were made by taking filter lifts of each large plate and placing the filters on plates containing fresh dropout media lacking tryptophan, leucine, histidine and containing 20 mM 3-AT. The replica plates were incubated at 30°C for 2 – 3 days and the filters were then treated with liquid nitrogen and stained in Z-Buffer for 8 hours. After staining the filters were dried in a fume hood over-night. Filters are shown in Appendix I.

3.2.6 – Yeast PCR and identification of interaction partners

Results of the yeast two-hybrid screen are summarised in Tables 3.2 and 3.3 and in Appendix II. The stained filters were used to identify colonies on the replica plates that were expressing the LacZ reporter gene. Once the colonies had started to grow on the replica plates prey inserts were picked using a p200 pipette tip and placed in two groups, strong blues or weak blues (Appendix II). A small amount of the yeast picked was used to inoculate a PCR reaction and the remainder used to streak an agar plate containing dropout media in order to generate a master plate.

Totals	All Clones	Positives	Out of Frame	No Insert	No Match	Misc.
Numbers	113	33	63	5	8	4
Percentages	100%	29%	56%	4%	7%	4%

Table 3-2: Summary of clones sequenced from yeast two-hybrid screen

Number	Name	Clones Identified
1	Nucleoside diphosphate kinase B	1, 2, 4, 31, 37, 56, 59, 62, 67, 82, 97
2	EID-1	15, 17, 26, 46, 66, 89
3	Activator of S-phase kinase (ASK)	6, 74, 99, 98, 103
4	CDC37	5, 7, 65
5	Mcl-1	31, 45, 85
6	Bcl-2	29
7	Thymic dendritic cell derived factor 1	48
8	Hypothetical Protein from BRCA Region	87
9	CDC2L5 (isoform 2)	100
10	Rpl13a	112

Table 3-3: Summary of positive clones pulled out of yeast two-hybrid screen.

The primers GADF1 and GADR1 were used to PCR all the prey inserts from the colonies picked as outlined in Chapter 2. PCR products were run out on a 1 % agarose gel and were cleaned up using a Qiagen PCR clean-up kit. Where possible these bands were sequenced directly using the GADF1 primer that had been used for PCR, and the sequence analysed. Prey cDNA sequences were identified using the BLAST tool to align the sequence to those available on the nucleotide database on the NCBI web site.

Five of the clones picked (4%) contained no cDNA insert. Of these 5 clones 4 were represented as strong blue colonies and 1 was represented by a weak blue colony (Appendix II). These were most likely to have had spontaneous mutations that allowed activation and expression of the reporter genes. Three of the clones contained 3'UTR sequences that would not normally be expressed as protein and one of the clones contained a cDNA sequence that had been inserted 3' - 5' (Table 3.2 and Appendix II). As a result these clones were eliminated as potential interaction partners. The open reading frame (ORF) of each cDNA pulled out of the screen was also checked to ensure that it was in-frame with the ORF of the upstream GAL4-AD. Of the 113 clones picked 63 (56%) of them were not in the same ORF as the upstream GAL4-AD (Table 3.2 and Appendix II) and these were automatically eliminated as a potential interaction partners. Eight of the clones (7%) matched no sequence in the database and translating the sequence in the same frame as the upstream GAL4-AD suggested that the fusion protein expressed was a nonsense protein. Ten potential interaction partners for tBid were identified from the remaining prey cDNA inserts analysed. These initial targets are outlined in Table 3.3.

Two of these ten potential partners for tBid were Bcl-2 family members. There was one clone containing a *bcl-2* cDNA (clone 29), a protein that has already been reported to interact with tBid. The other Bcl-2 family member identified was Mcl-1, another anti-apoptotic protein. The three clones identifying Mcl-1 (Table 3.3) contained independent cDNA inserts that started in different regions of the cDNA (Figure 3-8). Nucleoside diphosphate kinase B (NDK-B) was identified in more clones (11) than any other cDNA (Table 3.3). However all these clones contained the same cDNA sequence and had 90 bp of 3'UTR upstream of the start codon. There

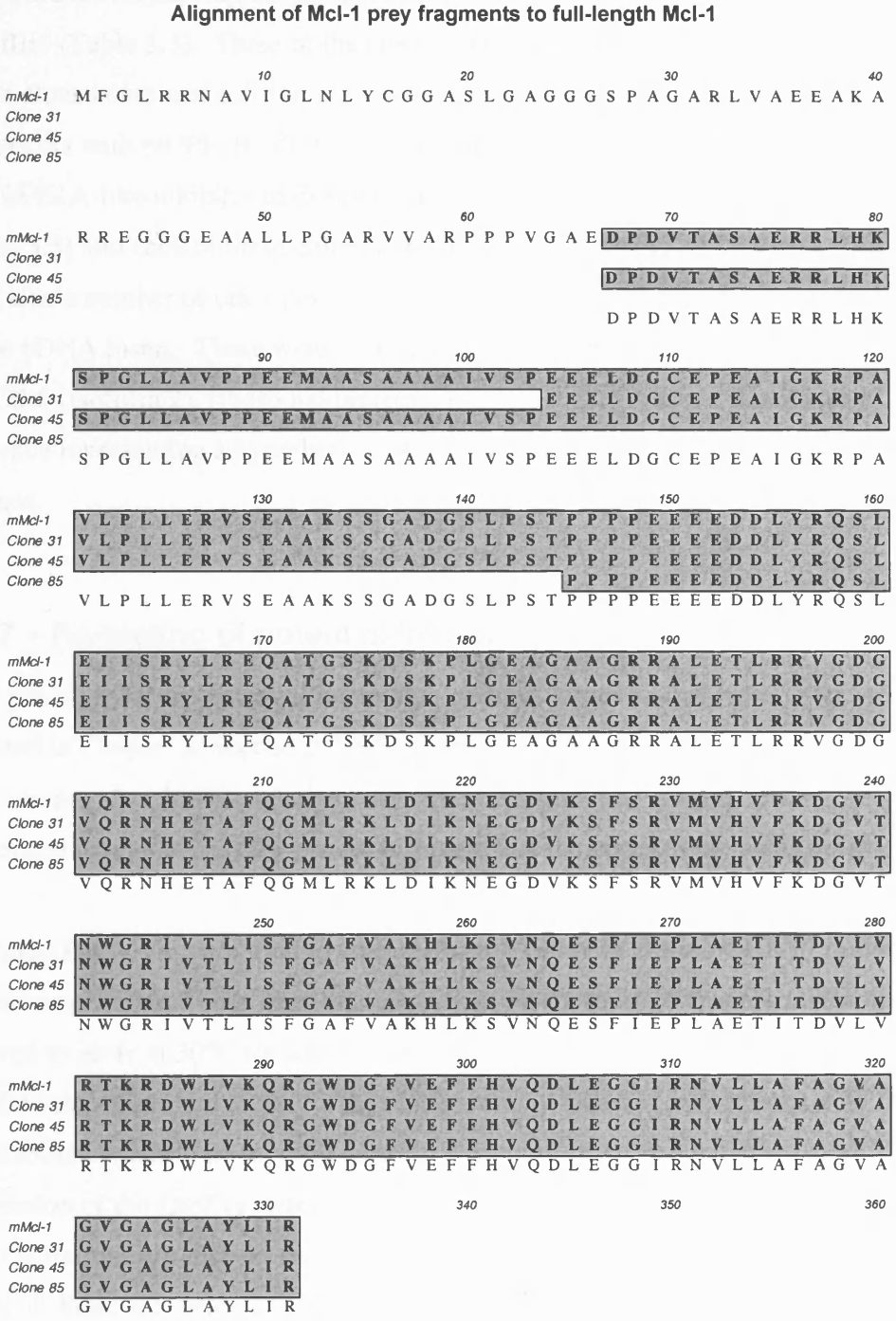


Figure 3-8: Alignment of Mcl-1 prey fragments from yeast two-hybrid assay.

were 4 clones identifying the protein activator of S-phase kinase (ASK) as interacting with tBid (Table 3.3). Three of the clones were represented by one cDNA while the fourth clone contained a different cDNA. Three clones were identified CDC37 cDNA. As with NDPK-B, all the clones contained the same cDNA sequence. Murine E1A-like inhibitor of differentiation-1 (EID-1) was identified in 6 clones (Table 3.3) and each of these clones contained independent cDNA inserts. There were also a number of other potential interaction partners that were represented by a single cDNA insert. These were murine thymic dendritic cell-derived factor 1, CDC2L5 (isoform 2), ribosomal protein L13a, and a cDNA insert that matched to a sequence representing a hypothetical protein from the BRCA2 chromosomal region in humans.

3.2.7 – Re-testing of potential interaction partners

Prey plasmids for the potential interaction partners were isolated from yeast as outlined in Chapter 2, Section 2.2.3.5. Restriction digestion analysis was used to confirm that all colonies contained the same cDNA insert. All prey inserts were again sequenced using the GADF1 primer to ensure that the correct insert was obtained.

Miniprep DNA was then used to co-transform Y190 with either pGBKT7 (empty bait vector) or pGtBid. These were then plated out on appropriate dropout media and allowed to grow at 30°C for 2 to 3 days until colonies appeared. Filter lifts were then taken of each plate and tested for *LacZ* expression. Genuine interactions with tBid in yeast should result in no reporter activation in transformations with the pGBKT7 but expression of the *LacZ* reporter in transformations with pGtBid. If both transformations are blue on staining with Z-Buffer, this indicates a false positive as a result of direct activation of the reporter by the prey fusion protein or interaction with the GAL4-DBD. However if neither transformations are blue on staining with Z-Buffer this indicates a spontaneous mutation within the specific Y190 clone originally identified allowing expression of the reporter genes, and not as a result of an interaction between the bait and prey fusion proteins.

Representative filter lift assays for the retest transformations are shown in Appendix III and summarised in Table 3.4. The retest assays identified NDK-B, CDC37, Bcl-2,

Mcl-1 and EID-1 clones as interacting with the tBid bait in yeast. The ASK and CDC2L5 proteins were both able to activate the reporter genes in the absence of the bait protein tBid while the thymic dendritic cell factor 1, ribosomal protein L13a and the hypothetical protein (from BRCA2 chromosomal region) did not appear to be able to switch on the reporters at all. This gave five *true* positives that interacted with the GAL4-DBD-tBid fusion protein in Y190.

3.2.8 – Constructs for the GST-pull down assay

Human cDNAs for each protein were obtained and cloned by PCR into pcDNA3.1-HA (Fig. 3-9A). This vector is pcDNA3.1 myc-HIS-(B) (Invitrogen) that has been engineered to contain a HA epitope tag that would be fused to the N-terminus of recombinant proteins. Details of the cloning procedure are outlined in Chapter 2, Section 2.2.2.2.

Human Bid and tBid were cloned by PCR into the pGEX-6P2 (Amersham) GST-fusion vector (Fig. 3-9B).

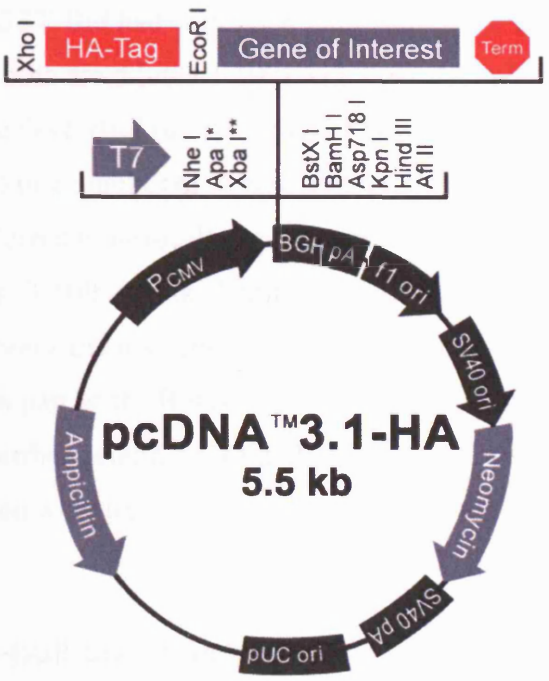
3.2.9 – Preparation of GST fusion proteins

The pGEX-Bid and pGEX-tBid were used to transform the *E. coli* BL21 host strain. Cultures for GST-fusion protein expression were prepared and IPTG added to a final concentration of 100 μM to induce expression of the fusion proteins. Cultures were allowed to grow for 2 hours after addition of IPTG and were then harvested by centrifugation. Pellets were washed once and resuspended in ice-cold PBS. Sonication was used to break open the cells and the fusion proteins were purified using glutathione sepharose beads. The sepharose beads were resuspended in a final volume of 1 ml PBS and stored at 4°C. Bead fractions were run on an SDS-PAGE denaturing gel to determine efficiency of fusion protein production and to determine how much of the sepharose GST-fusion bead complex to use for pull-down assays. Gels were fixed and stained with Coomassie blue.

Number	Name	Retest Result
1	Nucleoside diphosphate kinase B	Positive
2	EID-1	Positive
3	Activator of S-phase kinase (ASK)	Auto-activation
4	CDC37	Positive
5	Mcl-1	Positive
6	Bcl-2	Weakly positive
7	Thymic dendritic cell derived factor 1	Negative
8	Hypothetical Protein from BRCA Region	Negative
9	CDC2L5 (isoform 2)	Auto-activation
10	Rpl13a	Negative

Table 3-4: Summary of retest results for positive clones identified in the yeast two-hybrid assay. Corresponding filter lift assays are shown in Appendix III.

A



B

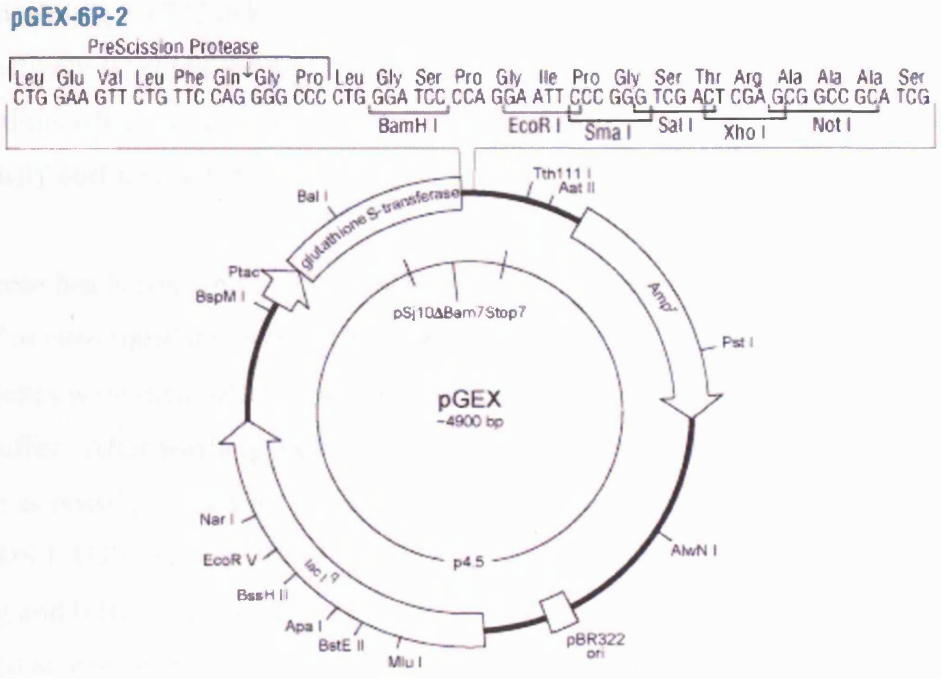


Figure 3-9: Vectors used for GST pull-down assay. Prey cDNAs cloned into pcDNA3.1-HA (A) were used for *in vitro* translation while GST-fusion proteins were generated by cloning bait proteins down-stream of GST in pGEX-6P-2 (B).

GST and the GST-fusion proteins GST-Bid and GST-tBid are shown in Figure 3-10A. Both GST and GST-Bid had very good induction in the BL-21 *E. coli* giving a high yield of protein (both are diluted 1:100) with little degradation of the fusion protein GST-Bid. The GST-tBid fusion construct however did not give as good a yield and also resulted in a number of degradation products. A similar SDS-PAGE gel was run and transferred to nitrocellulose membrane for Western blotting using an anti-Bid antibody (Fig. 3-10B). This identifies both the GST fusion proteins as containing the Bid protein and also confirms that the degradation products in the GST-tBid lane contain part of the Bid protein. These fusion proteins were used in pull-down assays to further determine if the tBid interaction partners identified in the yeast two-hybrid screen were true interaction partners for the protein.

3.2.10 – The GST-pull down assay

Using the pcDNA3.1-HA constructs for the human proteins, detailed in section 3.2.8 above, all potential interaction partners were *in vitro* translated in the presence of [^{35}S]-Met using a T7 Quick-coupled *in vitro* translation kit from Promega. Radioactively labelling the proteins allows for sensitive detection of even weak interactions when using the GST-pull down assay. This procedure has already been previously outlined in Figure 3-2.

Sepharose beads containing GST alone, GST-Bid, and GST-tBid were incubated with 2 µl of *in vitro* translated product and mixed by rotation at 4°C for 2 hours. Complexes were then pelleted in a microfuge and washed 6 times with 1 ml ice-cold lysis buffer. After washing, beads were pelleted with as much of the supernatant and residue as possible removed. Sample buffer was added to each tube and then resolved on a SDS-PAGE gel. Gels were stained using Coomassie blue to check for equal loading and following drying were exposed to a phosphorimager screen overnight. Exposed screens were scanned the following day. Results are shown in Figure 3-11. The first lane in each image contains 10% of the *in vitro* translated prey protein used in the pull-down. The other lanes show the amount of prey protein pulled down by GST alone, GST-Bid fusion or GST-tBid fusion when the prey protein and fusion proteins are incubated together.

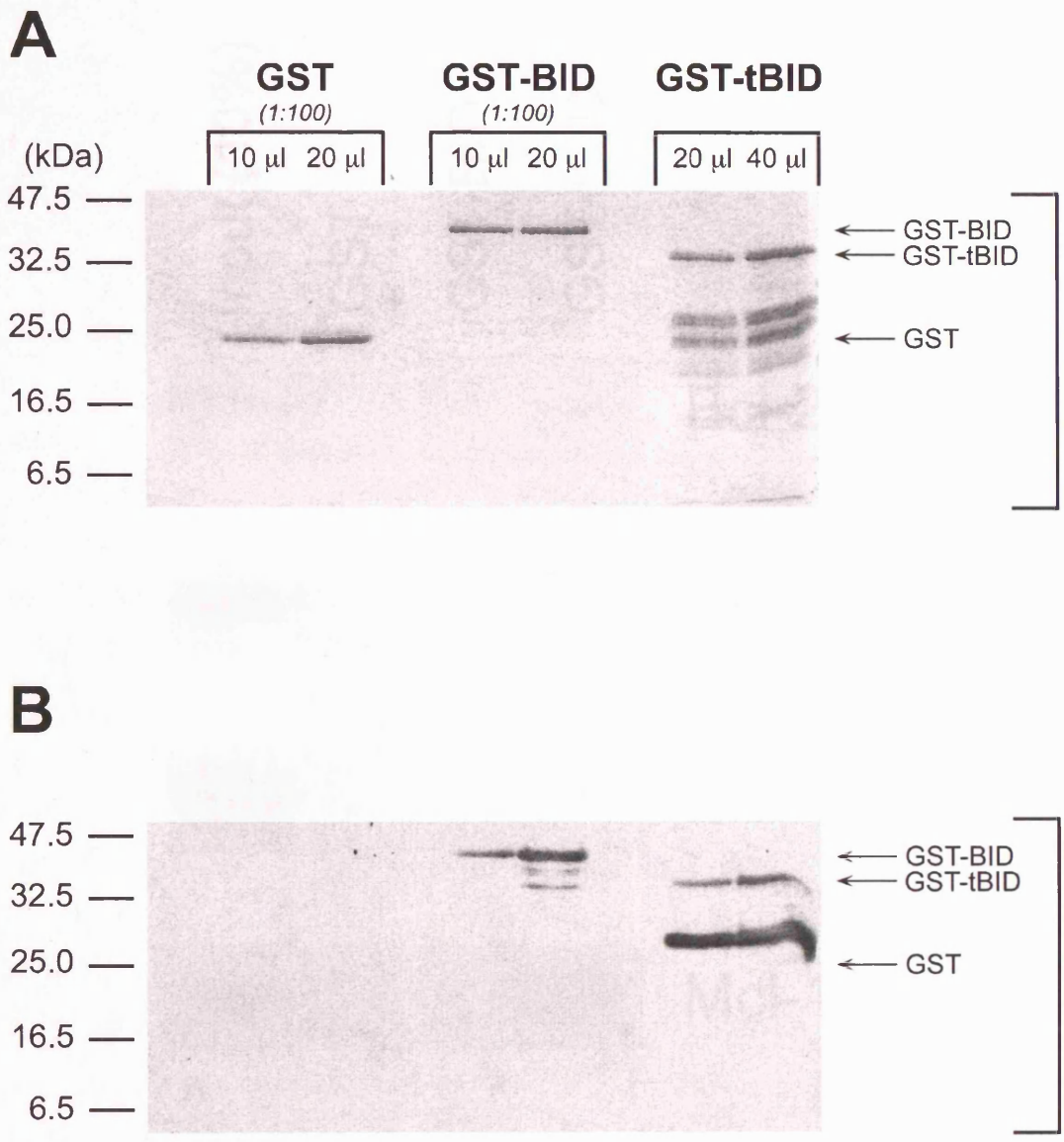


Figure 3-10: GST-fusion proteins used for pull-down experiments. (A) A Coomassie stained gel showing the loading of GST, GST-Bid and GST-tBid purified fusion proteins. (B) A Western blot corresponding to the gel shown in (A) using an anti-Bid antibody.

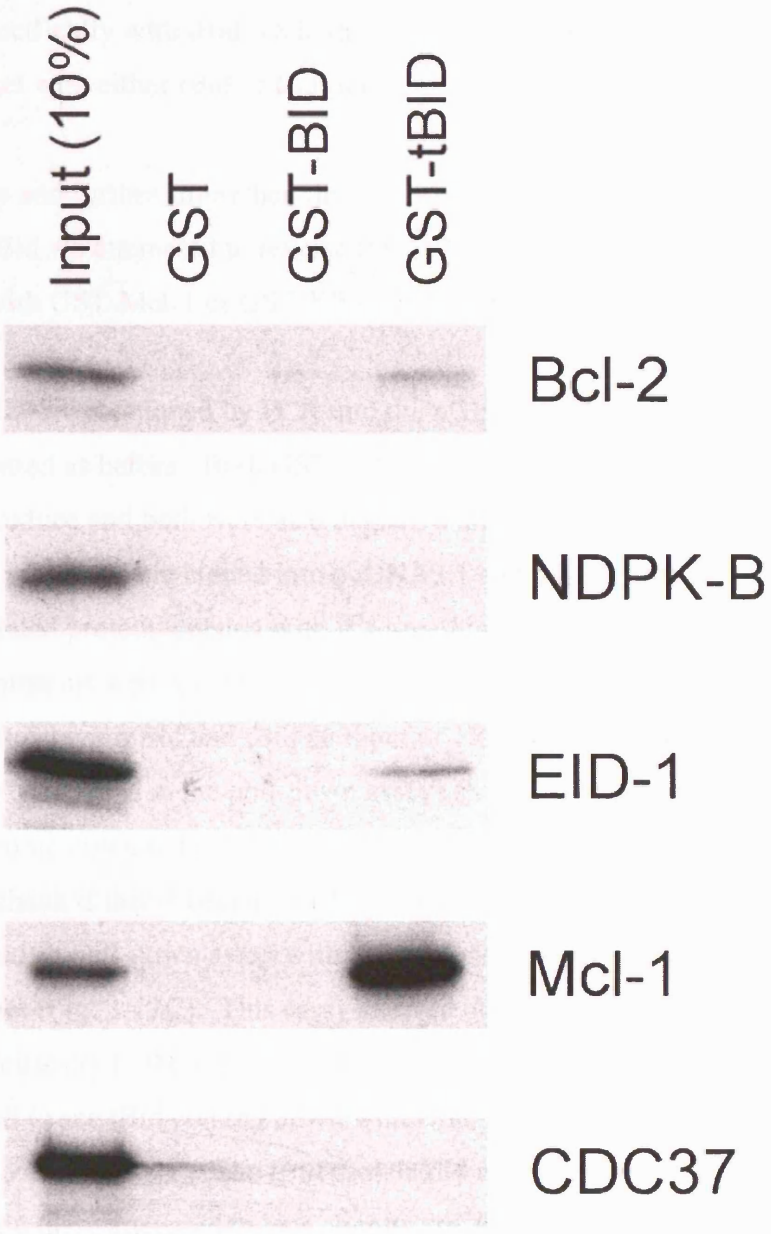


Figure 3-11: GST pull-down assay using GST-Bid and GST-tBid. Prey constructs in vitro translated in the presence of [^{35}S]-methionine were incubated with the GST fusion proteins to test for interaction between the bait and prey proteins.

As expected both Bcl-2 and Mcl-1 interact with tBid, however it is interesting that they do not interact with full length Bid. The interaction between tBid and Mcl-1 also appears to be stronger than that between tBid and Bcl-2. EID-1 also shows an ability to interact specifically with tBid while the other two proteins, NDPK-B and CDC37, did not interact with either tBid or Bid in this assay.

In order to try and further strengthen the case for the interaction of both Mcl-1 and EID-1 with tBid we attempted to reverse the pull-down assay already by pulling down Bid or tBid with GST-Mcl-1 or GST-EID-1 fusion proteins.

Mcl-1 and EID-1 were cloned by PCR into the pGEX-6P2 vector and GST-fusion proteins prepared as before. Both GST-EID-1 and GST-Mcl-1 proved to be quite difficult to produce and both were also degraded during the preparation (Fig. 3-12A). Bid and tBid proteins were cloned into pcDNA3.1 and this was then used to generate *in vitro* translated protein labelled with [^{35}S]-methionine. GST-alone, GST-Mcl-1 or GST-EID-1 proteins were used to pull down radioactively labelled Bid or tBid. In the case of both full-length Bid and tBid an input of 1% was used. This is much lower than the 10% input used in the pull-down assays shown in Figure 3-11. The [^{35}S]-Bid appeared to come down in both the GST and GST-fusion lanes (Fig. 3-12B, top panels). To check if this is because of Bid binding to GST or to the glutathione sepharose beads a pull-down assay with beads alone or beads containing bound GST was carried out (Fig. 3-12C). This assay showed quite clearly that Bid appeared to bind non-specifically to the sepharose beads. For the pull-down assay with [^{35}S]-tBid it was difficult to see tBid coming down with either GST or the GST-fusion proteins (Fig. 3-12B, bottom panels). The tBid protein did appear to be binding slightly to the GST beads and there appeared to be a slightly stronger band in the GST-fusion lane, although this result was difficult to reproduce. The tBid in general was difficult to see and may be as a result of weak binding or due to problems with the stability of the tBid protein in the assay.

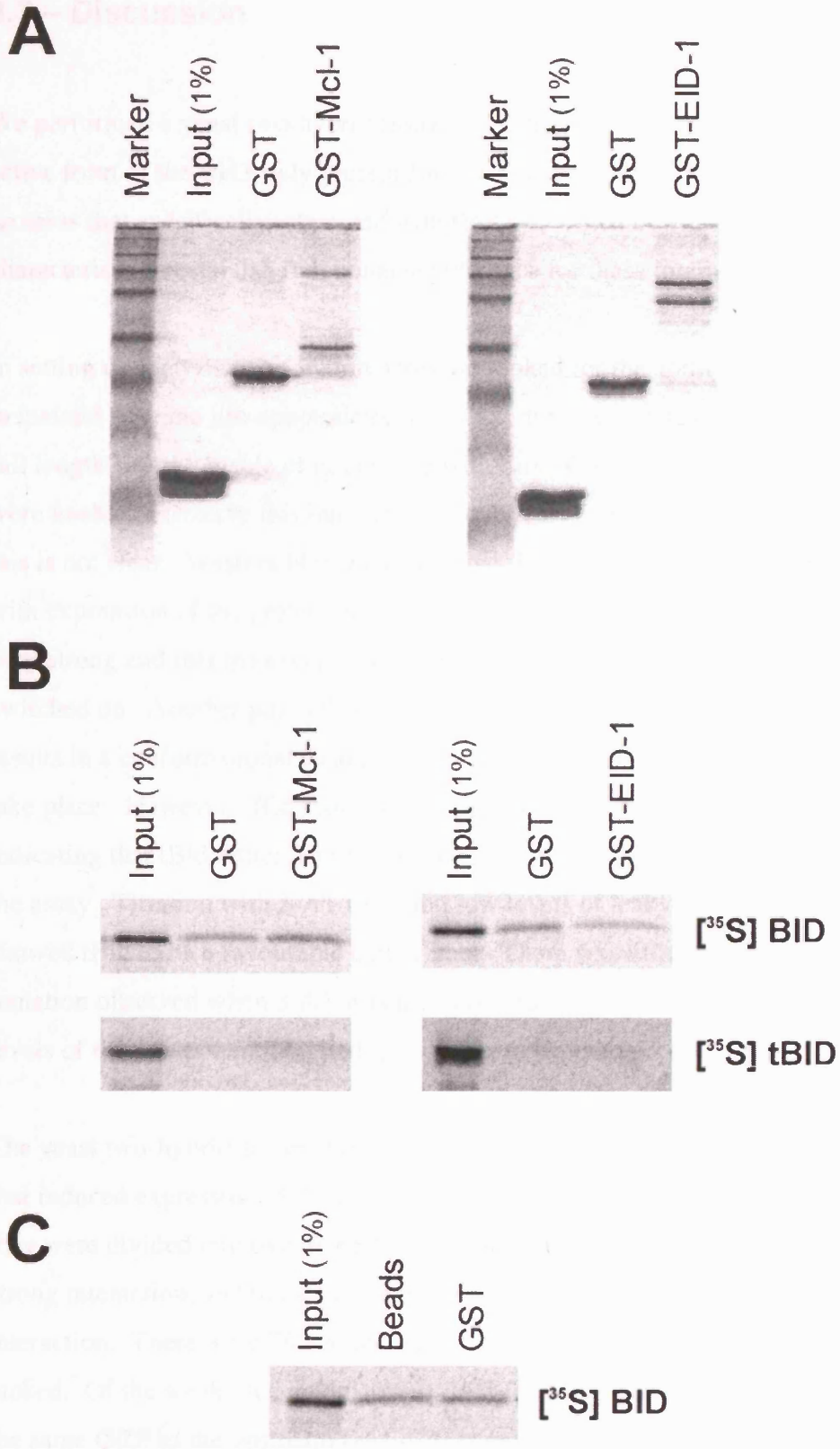


Figure 3-12: GST pull-down assays using GST-Mcl-1 or GST-EID-1.

3.3 – Discussion

We performed a yeast two-hybrid assay to identify novel interaction partners for the active form of the BH3-only protein Bid. We successfully identified a number of proteins that specifically interacted with tBid and which now need to be further characterised to establish functional significance for these interactions.

In setting up the yeast two-hybrid assay we looked for the ability of both Bid and tBid to interact with the pro-apoptotic protein Bax. It has previously been reported that full length Bid is capable of interacting with Bax (Wang et al., 1996), however we were unable to observe this interaction in our yeast two-hybrid assay. The reason for this is not clear. Western blots indicated that there did not appear to be a problem with expression of the protein in yeast and so it is possible that the interaction is not very strong and that the assay was not sensitive enough to allow reporters to be switched on. Another possibility is that fusing full length Bid to the GAL4-DBD results in a conformational change that is unfavorable for the Bid-Bax interaction to take place. However, tBid showed a strong interaction with Bax in this assay indicating that tBid rather than the full-length protein would be a more suitable bait in the assay. Titration with 3-AT to inhibit low levels of leaky *HIS3* expression also showed tBid to be a favourable bait protein. There was little toxicity or spontaneous mutation observed when 3-AT was incorporated into the media and reasonably low levels of the compound inhibited growth due to leaky *HIS3* expression.

The yeast two-hybrid screen itself identified 113 clones that contained prey cDNAs that induced expression of the reporter genes in Y190. As the clones were picked they were divided into two groups. Those that gave a strong blue colour, indicating a strong interaction, and those that gave a weaker blue colour, indicating a weak interaction. There were 78 strong blue colonies picked and 35 weak blue colonies picked. Of the weak blue colonies only 15% (5 of 33) of cDNA constructs were in the same ORF as the upstream GAL4-AD compared with 36% (28 of 78) of the strong blue cDNA constructs that were in the same ORF as the upstream GAL4-AD. All prey plasmids containing a cDNA insert that was not in the same ORF as the upstream activation domain were automatically eliminated as potential interaction

partners. A number of other clones could also be eliminated after initial sequence analysis. One of the clones contained no insert in the prey vector and probably a spontaneous mutation within the yeast to allowed activation of the reporters, while a number of other inserts contained only UTR sequence that is not normally expressed as protein in cells, hence these clones were automatically eliminated.

The 33 clones identified as possible interaction partners contained cDNAs that encoded 10 different proteins. Of these proteins five were pulled out of the screen only once. One of these cDNA inserts encoded the anti-apoptotic protein Bcl-2. This was the only one of these proteins to retest positive. The activator of S-phase kinase (ASK) protein was pulled out a number of times but was later identified as being able to activate the reporter genes in the absence of tBid. This resulted in the elimination of this protein as a potential interaction partner. The remaining prey cDNA constructs identified, NDPK-B, EID-1, CDC37 and Mcl-1, all retested positive in the yeast two-hybrid assay and were considered good candidates as potential novel interaction partners for tBid.

GST pull-down assays were used to confirm the interaction of the bait and prey proteins outside of yeast. GST-Bid and –tBid fusion proteins were generated in BL21 *E. coli* and purified using glutathione sepharose beads. GST-Bid was relatively easy to produce while GST-tBid was more difficult and expression of this protein in the bacterial host strain resulted in some degradation of the fusion protein. Prey proteins were *in vitro* translated in the presence of [^{35}S]-methionine and then incubated with the fusion proteins to check for interaction.

As Bcl-2 is a known interaction partner for tBid we used this as a positive control for our assay. We were successfully able to pull-down Bcl-2 with tBid in this assay, however there did not appear to be any interaction between Bcl-2 and full-length Bid. A similar result is seen in the case of Mcl-1. Again tBid is specifically able to pull-down the Mcl-1 protein while the full-length Bid does not pull-down Mcl-1. It is interesting that in both the yeast two-hybrid and GST pull-down assays the interaction between tBid and Bcl-2 is much weaker than that between tBid and Mcl-1. This suggests that Mcl-1, rather than Bcl-2, is a preferred interaction partner for tBid. The

EID-1 protein was the only other protein that was seen to interact with tBid in both assays used. Again this interaction is specific for tBid. The fact that Bcl-2 does not interact with full-length Bid is surprising as this interaction has been reported previously using this assay. However tBid is the active form of the BH3-only protein and so should have a higher affinity for interaction with other Bcl-2 family members in this state.

Neither NDPK-B nor CDC37 appear to interact with the GST-Bid or -tBid fusion proteins in this assay. Although a number of clones identified both these proteins in the yeast two-hybrid assay, the cDNA insert identified was exactly the same for each case of NDPK-B and CDC37. In the case of NDPK-B, all cDNA inserts contained the last 90 bp of 5'UTR before the initiation codon. This adds an extra 30 amino acids to the N-terminus of the protein that may result in an altered confirmation and allow interaction with the tBid fusion protein and subsequent activation of the reporter genes in yeast. When these 30 amino acids are not present (as in the case with the GST pull-down assay) and the protein is in its normal conformation this interaction should not occur. Similarly with CDC37, as there was only one cDNA identified representing the protein, the interaction may be dependent on the confirmation achieved when only this exact part of the protein is fused to the GAL4-AD. In contrast, both for Mcl-1 and EID-1, all clones identified contained inserts starting at different regions in the mRNA resulting in slightly different fusion proteins that consistently interact with the bait.

It is worth noting however that at least in the case of CDC37 there maybe other reasons for its failure to interact outside of a cellular system. CDC37 has a number of different roles to play within the cell. One of these relates to its ability to act as a chaperone in a complex with a number of other proteins (Kimura et al., 1997). This protein complex involves amongst other proteins the heat-shock protein 90 (Hsp90) (Pratt et al., 1999). It has been shown in the literature that Hsp90 can be co-immunoprecipitated with Bid from NIH3T3 fibroblasts (Zhao and Wang, 2004). Thus if CDC37 can interact with Bid or tBid it may interact as part of a complex with other proteins. Heat-shock proteins in yeast may be able to complex with the GAL4-AD-CDC37 fusion protein and binding to the bait protein and activation of the

reporter genes. In the GST pull-down assay there are no other proteins present, thus CDC37 maybe unable to interact with Bid or tBid without some other chaperone complex proteins present.

We have identified two novel interaction partners for tBid. The anti-apoptotic Bcl-2 family member Mcl-1, and the recently identified E1A-like inhibitor of differentiation (EID-1).

Although there is little published on the role of EID-1, there is no real evidence to suggest that it may have a role in apoptosis. Overexpression of EID-1 shows that it localises to both the cytoplasm and the nucleus (Bavner et al., 2002), and cells overexpressing the protein do not appear to have any alteration in cell-cycle and are not prone to apoptosis (MacLellan et al., 2000; Miyake et al., 2000). Its function to date has been described as being primarily nuclear, interacting with proteins such as pRb to maintain cells in a proliferate state, and downregulation of the protein appears to be required to allow for differentiation of fibroblasts to proceed (MacLellan et al., 2000; Miyake et al., 2000).

Mcl-1 is a protein that is known to protect cells from apoptosis (Reynolds et al., 1994; Zhou et al., 1997). It is a Bcl-2 family member that is localised to the mitochondria (Akgul et al., 2000) and that has been shown to have an essential role in early development and in the differentiation and maintenance of haematopoietic cells (Opferman et al., 2003; Rinkenberger et al., 2000). However there is little work characterising Mcl-1s ability to interact with BH3-only members of the Bcl-2 family and this makes it an excellent candidate as a potential interaction partner for tBid.

4 - Mcl-1 Cleavage

4.1 – Introduction

Mcl-1 is a Bcl-2 family member that was first identified as an early response gene upregulated in ML-1 cells in response to the differentiation agent PMA (Kozopas et al., 1993). It has since been shown to have an important role early in development (Okita et al., 1998; Rinkenberger et al., 2000) as well as in differentiation and maintenance of haematopoietic cells (Opferman et al., 2003). It is thought that the role of Mcl-1 as an anti-apoptotic Bcl-2 family member is paramount to its function in differentiation and development (Craig, 2002).

4.1.1 – Mcl-1 structure

As a Bcl-2 family member, Mcl-1 contains all BH-1, -2 and -3 homology domains (Kozopas et al., 1993). It is much larger than other Bcl-2 family members, containing a large N-terminal region consisting of two PEST sequences. PEST sequences are regions within proteins that are rich in the amino acids proline (P), glutamine (E), serine (S) and theronine (T) and are thought to target a protein for rapid proteasome mediated degradation within the cell (Rechsteiner and Rogers, 1996). Mcl-1 is known to have a short half-life (Akgul et al., 2000; Cuconati et al., 2003; Iglesias-Serret et al., 2003) and it has been assumed that these PEST regions are responsible for this short $t_{1/2}$. Like many Bcl-2 family members Mcl-1 contains a transmembrane domain at its C-terminus. This localises the protein to membranes within the cell and in particular Mcl-1 appears to be localised to the mitochondrial membrane (Akgul et al., 2000). Here it can interact with other Bcl-2 family members and help prevent activation of pro-apoptotic proteins that result in release of mitochondrial proteins and subsequent progression of apoptosis.

4.1.2 – Mcl-1 and apoptosis

Since its discovery Mcl-1 has been shown to delay apoptosis in a number of cell types including CHO cells, and various haematopoietic cell types (Jourdan et al., 2003; Pedersen et al., 2002; Reynolds et al., 1996; Zhang et al., 2002; Zhou et al., 1997). By comparison with Bcl-2 however Mcl-1's anti-apoptotic potential is not as potent (Reynolds et al., 1996), and until recently little was known as to how Mcl-1 functions in protecting cells from apoptosis. Early data on Mcl-1 showed a role for this protein in protecting haematopoietic cell types. Mcl-1 transgenic mice show reduced apoptosis within haematopoietic cell populations (Zhou et al., 2001), and Mcl-1 was shown to be the primary anti-apoptotic protein in neutrophils (Moulding et al., 2001). There is also much data detailing regulation of Mcl-1 expression in the presence or absence of cytokines including IL-6 and GM-CSF (Chao et al., 1998; Jourdan et al., 2003; Puthier et al., 2001), and Mcl-1 appears to have a role to play in the pathogenesis of multiple myeloma (MM) (Derenne et al., 2002; Jourdan et al., 2003; Puthier et al., 2001; Zhang et al., 2002).

However, a number of papers published recently shed light on how Mcl-1 may function to protect cells from apoptosis. UV induced apoptosis of HeLa cells has been shown to require loss of Mcl-1 for apoptosis to proceed (Nijhawan et al., 2003). Overexpression of Mcl-1 in these cells results in complete inhibition of apoptosis. This data also shows that Mcl-1 acts upstream of another anti-apoptotic Bcl-2 family member, Bcl-X_L. Overexpression of Mcl-1 is able to prevent translocation of the pro-apoptotic Bax to the mitochondria. However, overexpression of Bcl-X_L is unable to prevent this translocation, but does inhibit apoptosis by preventing Bax from forming large molecular weight homo-oligomers that are required for release of mitochondrial proteins involved in apoptosis. Thus once HeLa cells have been treated with UV radiation, translation of Mcl-1 is inhibited. The remaining Mcl-1 in the cell is degraded by the proteasome and this then allows pro-apoptotic Bcl-2 family members to translocate to the mitochondria (Nijhawan et al., 2003). Adenoviral-infected cells also result in destruction of Mcl-1, which again appears critical for apoptosis to proceed in this setting (Cuconati et al., 2003). This data shows that Bak interacts specifically with Mcl-1 in HeLa cells. It also suggests that the loss of Mcl-1 during infection is not just a consequence of apoptosis but a defining event in the initiation of

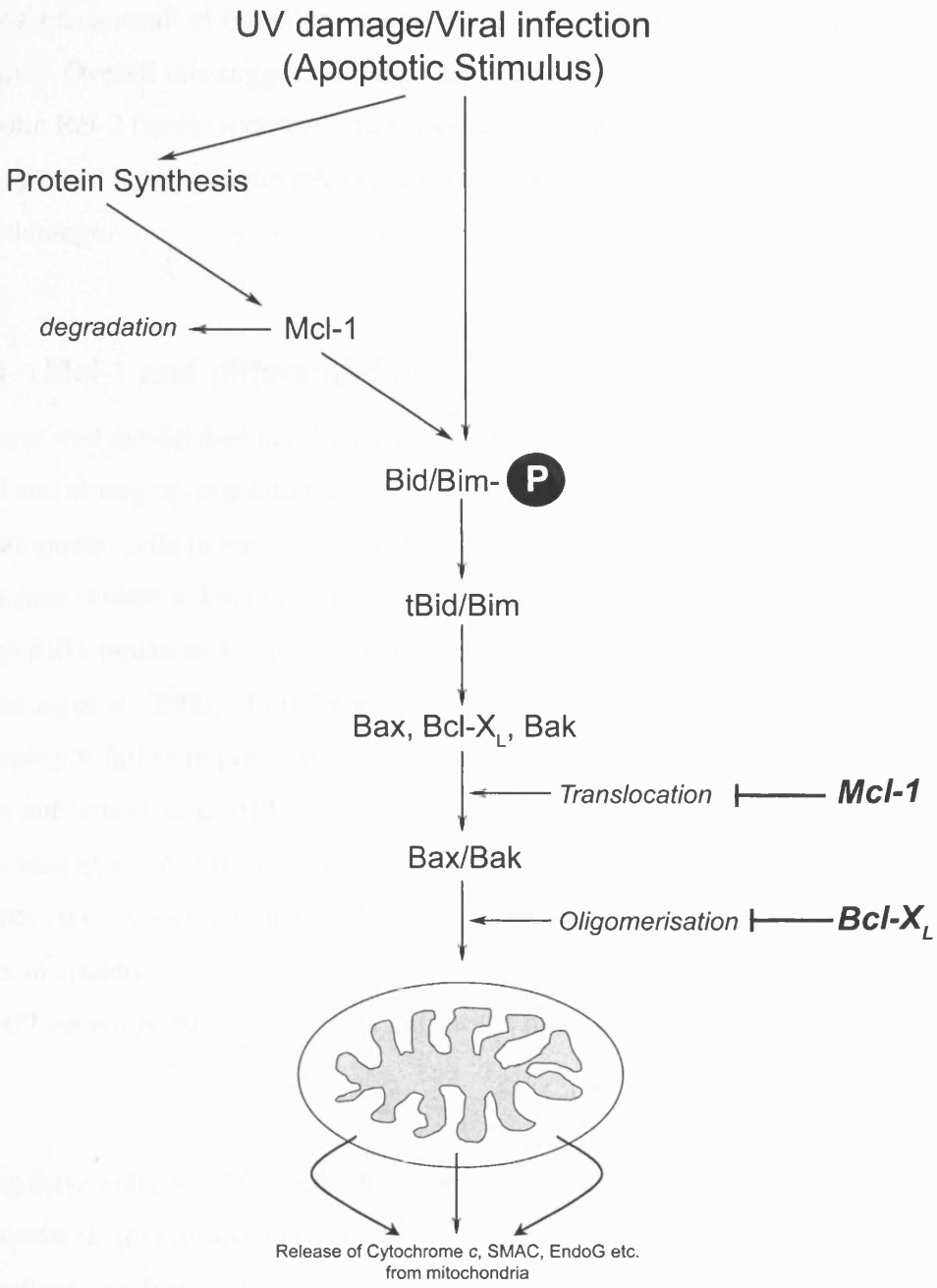


Figure 4-1: Mcl-1 and apoptosis. As outlined in Section 4.1.2 Mcl-1 is degraded rapidly in response to DNA damage and appears to be an early event in this pathway. This can allow for activation of BH3-only family members although this activation may independent of Mcl-1 elimination. Overexpression of Mcl-1 (shown in ***bold italics***) appears to prevent translocation of other Bcl-2 family members to the mitochondria. This activity of Mcl-1 is upstream of Bcl-X_L activity, which cannot prevent translocation of Bcl-2 family such as Bax, but can prevent their ability to induce release of mitochondrial proteins through homo-oligomerisation.

apoptosis as a result of infection, as a result of E1A initiated DNA damage type response. Overall this suggests an important role for Mcl-1 in interacting with pro-apoptotic Bcl-2 family members, and also that elimination of Mcl-1 is an important primary event leading to the release of cytochrome *c* for apoptosis in response to DNA damage.

4.1.3 – Mcl-1 and differentiation

It is now well established that Mcl-1 has an important role to play in differentiation. Rapid and strong up-regulation of Mcl-1 at the level of both mRNA and protein in haematopoetic cells in response to differentiation agents has been well documented. Anti-sense studies to knockdown Mcl-1 protein levels at this early stage in response to such differentiation agents results in rapid apoptosis and an inability to differentiate (Moulding et al., 2000). Initial attempts to develop an Mcl-1 knock-out mouse show that embryos fail to implant and die by between E4.0 and E5.0. A conditional Mcl-1 knock-out mouse generated was used to knockout Mcl-1 out of B- and T-cell lineages (Opferman et al., 2003). This results in the complete abolition of both B- and T-lymphocytes. Knocking out the Mcl-1 gene in mature B- or T-lymphocytes also results in apoptosis of these cells. This indicates that Mcl-1 is required not only for normal haematopoetic development but for the maintenance of mature haematopoetic cells.

During differentiation cells undergo a vast metamorphosis in terms of gene expression that results in alterations to cellular structure, function, and morphology. In committing to differentiation these changes require a reorganisation of chromatin structure to alter gene expression. Recent data has shown that in addition to changes in gene expression, caspase activation may also play an important role in differentiation (Arama et al., 2003; Fernando et al., 2002; Pandey et al., 2000; Weil et al., 1999; Zermati et al., 2001). This can result in a large stress placed upon the cell that needs to be managed very carefully. Certainly part of the role of Mcl-1 in differentiation and development would appear to be linked to its function as an anti-apoptotic protein. Stabilisation and protection of cells from inappropriate apoptosis is essential during the commitment to differentiation. In particular Mcl-1's apparent

role in protecting from DNA damage may be also relevant for differentiating cells as they undergo vast changes in their gene expression profile.

Having identified Mcl-1 as a potential interaction partner for the BH3-only protein tBid as outlined in Chapter 3, we were interested in looking for any changes to Mcl-1 during apoptosis. It is thought that Bid cleavage plays an important role in Fas mediated apoptosis of the Jurkat T-cell lymphoma cell line. By treating Jurkat cells with the Fas activating CH11 antibody we initially examined the effect of apoptosis on the Mcl-1 protein.

4.2 – Results

4.2.1 – Mcl-1 is cleaved during apoptosis in Jurkat cells

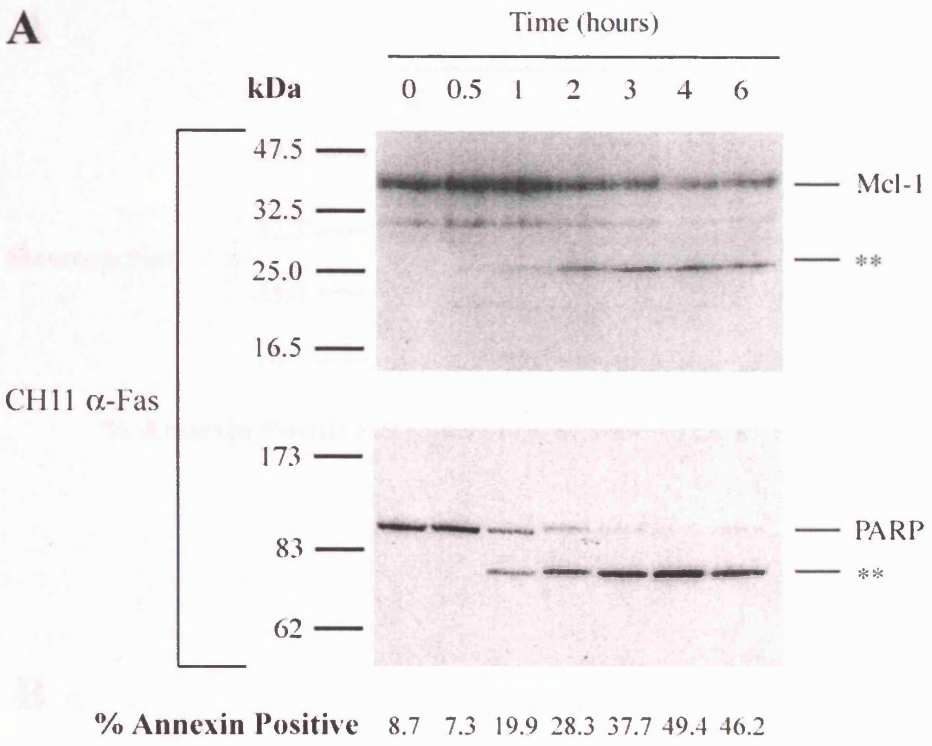
We initially observed that Mcl-1 was cleaved in Jurkat cells in response to treatment with the anti-Fas monoclonal antibody CH11. As shown in Figure 4-2A, Mcl-1 was cleaved from its full length 42 kDa form to a lower band migrating at about 25 kDa as detected by Western blotting. This time course showed that Mcl-1 cleavage is observed at the same time as PARP cleavage (Fig. 4-2A). In order to establish if this cleavage was upstream or downstream of caspase activity Jurkat cells were incubated with 75 μM of the pan-caspase inhibitor zVAD-fmk for 30 minutes before inducing apoptosis. Both Mcl-1 and PARP cleavage was completely inhibited over the 6-hour time course (Fig. 4-2B). This indicated that caspase activity is required for Mcl-1 cleavage. Inducing apoptosis with 1.0 μM staurosporine (Fig. 4-3A) or 25 μM etoposide (Fig. 4-3B) also resulted in a similar processing of Mcl-1.

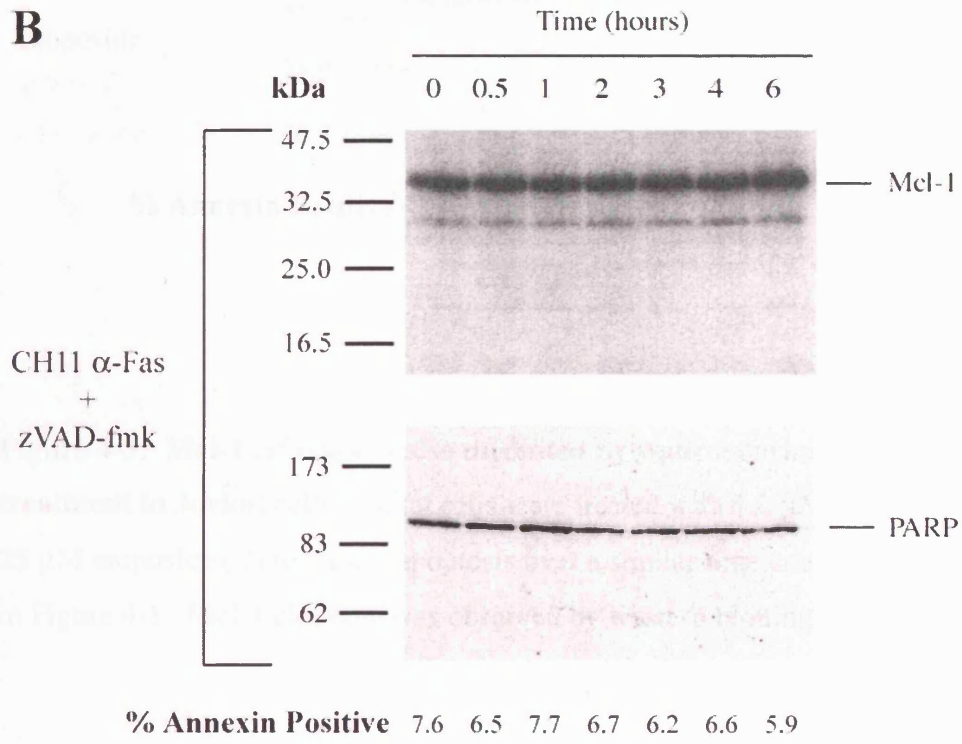
4.2.2 – Mcl-1 cleavage is mediated by caspases

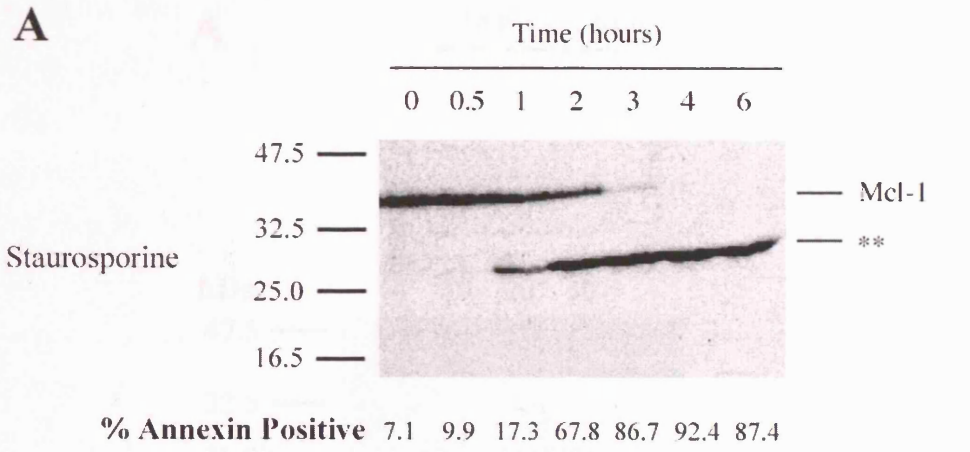
An *in vitro* system was set up to examine the processing of Mcl-1 more closely. Human and mouse Mcl-1 cDNAs were cloned by PCR into the pcDNA3.1-HA (Fig. 3-9A) vector as outlined in Chapter 2, Section 2.2.2.3. Both human (Fig. 4-4A) and mouse (Fig. 4-4B) HA-Mcl-1 were *in vitro* translated in the presence of [^{35}S]-methionine. The resulting radio labelled protein was incubated with a cytoplasmic lysate from untreated Jurkat cells or Jurkat cells treated with 300 ng/ml CH11 anti-Fas antibody for four hours. This lysate contains either inactive (i.e. non-apoptotic lysate) or active (i.e. apoptotic lysate) forms of caspases present in the cell. Treating the recombinant radiolabelled protein with increasing amounts of apoptotic lysate clearly showed the cleavage of both human and mouse Mcl-1 (Fig. 4-3A and 4-3B). Addition of 75 μM zVAD-fmk to the apoptotic lysate completely inhibited this cleavage as can be seen in the last lane in both Figures 4-4A and 4-4B.

These experiments show that human Mcl-1 is processed into three fragments while mouse Mcl-1 is processed into only two fragments. This suggests two cleavage sites

Figure 4-2: Mcl-1 is cleaved during Fas-mediated apoptosis in Jurkat cells. (A) Jurkat cells were treated with 300 ng/ml CH11 anti-Fas over a 6-hour time course. Western blots against Mcl-1 (top panel) of total cell lysate show cleavage of Mcl-1 while blots against PARP (bottom panel) are used as a marker of apoptosis. Cells were stained with anti-Annexin-PE and analysed by flow cytometry to show the progression of apoptosis. The percentage of Annexin positive cells at each time point is shown below each lane. (B) Cells were treated with 75 μM zVAD-fmk for 30 minutes prior to the addition of the CH11 anti-Fas antibody as outlined in (A). This shows complete inhibition of apoptosis and Mcl-1 cleavage.


A
Time (hours)
kDa
0 0.5 1 2 3 4 6
47.5
32.5
25.0
16.5
Mcl-1
**
CH11 α-Fas
173
83
62
PARP
**
% Annexin Positive 8.7 7.3 19.9 28.3 37.7 49.4 46.2


B
Time (hours)
kDa
0 0.5 1 2 3 4 6
47.5
32.5
25.0
16.5
Mcl-1
CH11 α-Fas
+
zVAD-fmk
173
83
62
PARP
% Annexin Positive 7.6 6.5 7.7 6.7 6.2 6.6 5.9



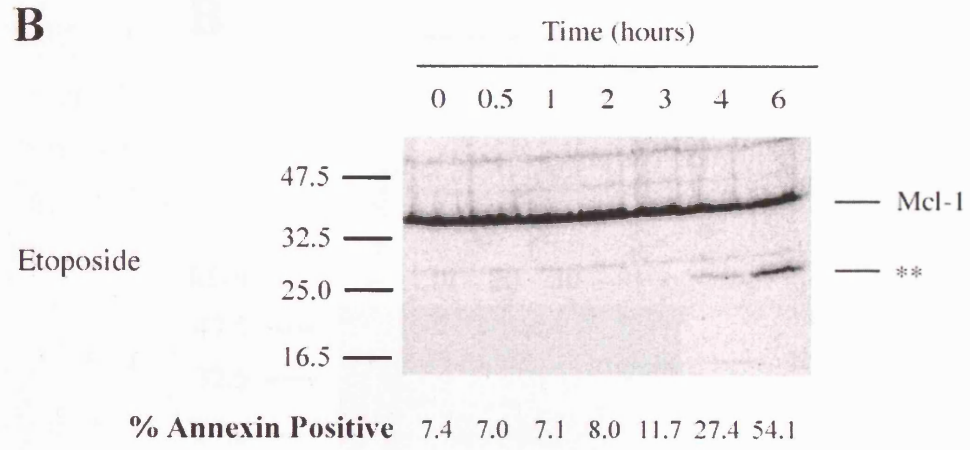


Figure 4-3: Mcl-1 cleavage is also mediated by staurosporine and etoposide treatment in Jurkat cells. Jurkat cells were treated with 1.0 μM staurosporine (A) or 25 μM etoposide (B) to induce apoptosis over a similar time course to that carried out in Figure 4-1. Mcl-1 cleavage was observed by western blotting.

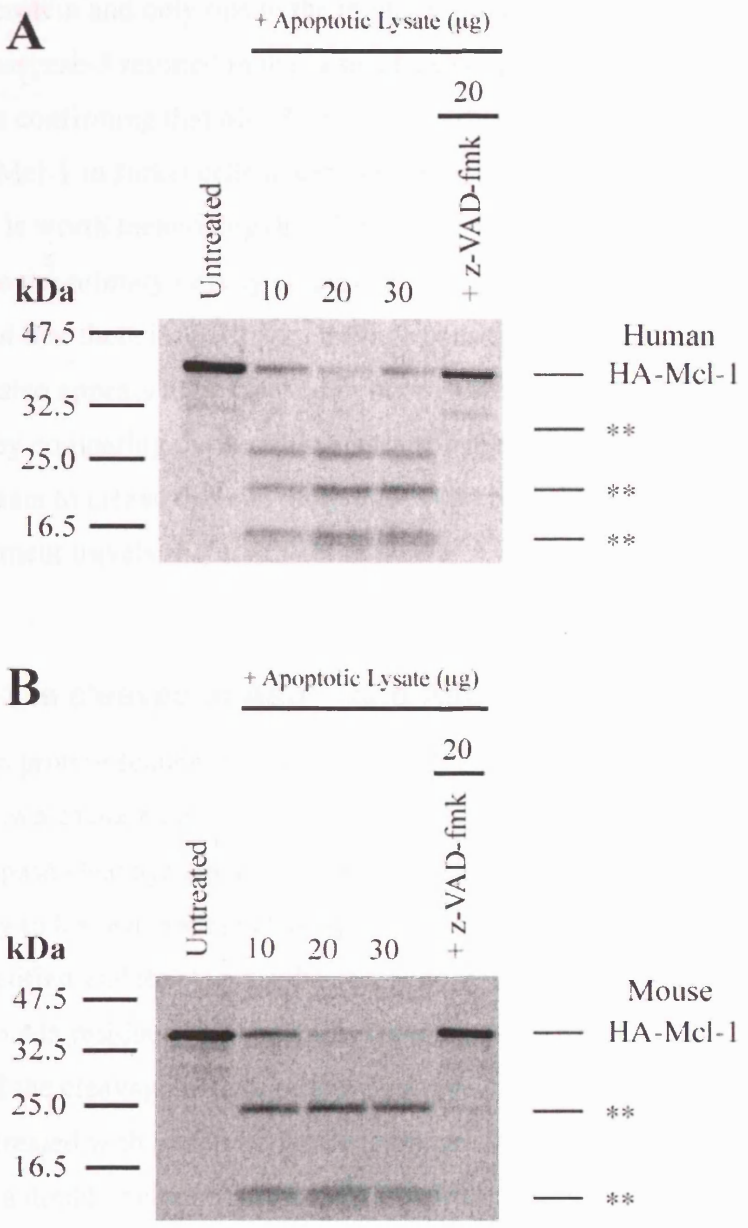


Figure 4-4: Human and mouse Mcl-1 can be cleaved in to a number of fragments. *In vitro* [^{35}S]-labelled human (A) and mouse (B) Mcl-1 were incubated with increasing amounts of apoptotic lysate for 2 hours at 37°C. Samples were resolved on an SDS-PAGE gel and exposed to a phosphorimager screen. The final lane in both gels shows that pre-incubating with 75 mM zVAD-fmk can abolish cleavage of the recombinant protein by apoptotic lysate, suggesting that caspases are responsible for the cleavage of Mcl-1.

in the human protein and only one in the mouse protein. Incubating human HA-Mcl-1 with active caspase-3 resulted in the same cleavage pattern as for apoptotic lysate (Fig. 4-5), thus confirming that Mcl-1 can be directly cleaved by caspases and that the processing of Mcl-1 in Jurkat cells undergoing apoptosis is due to caspase cleavage of the protein. It is worth mentioning that although caspase-3 can cleave Mcl-1 *in vitro*, this may not be the primary or only caspase involved in Mcl-1 processing *in vivo*. We have also noted that there is a caspase cleavage consensus site (DXXD) present in the HA tag. This also appears to be cleaved in our *in vitro* assays and can be easily seen in Figure 4-5 by comparing the smallest fragments present on the gel. Active caspase-3 appears to cleave this site more efficiently than the apoptotic lysate and so this small fragment travels slightly faster in lane 3.

4.2.3 – Mcl-1 is cleaved at Asp^{127} and Asp^{157}

Analysis of the protein sequence of the human Mcl-1 protein did not identify any obvious or known caspase cleavage sites. All Asp residues were examined as part of a potential caspase cleavage site according to the three residues N-terminal to Asp and their homology to known caspase cleavage sites. A number of candidate cleavage sites were identified and the Asp residues were subsequently mutated by site directed mutagenesis to Ala residues. As shown in Figure 4-6A, mutation of D127A or D157A altered the cleavage pattern of the wild-type (wt) HA-Mcl-1 radiolabelled protein when treated with apoptotic lysate (compare lanes 3 and 4 to lane 2). Generation of a double mutant (D127A, D157A Mcl-1) resulted in the complete abolition of Mcl-1 cleavage (Fig. 4-6A, lane 5). An identical cleavage pattern was also observed by using active caspase-3 (Fig. 4-6B). Thus the three fragments generated by cleavage of the human Mcl-1 molecule are (i) an N-terminal fragment from amino acids 1 to 127, (ii) a long C-terminal fragment from amino acids 128 to 350 (Δ127) and (iii) a shorter C-terminal fragment from amino acids 158 to 350 (Δ157) (Fig. 4-7).

Western blots using the Santa Cruz anti-Mcl-1 (S-19) antibody clearly show the Δ127 fragment (Fig. 4-2 and 4-3) but do not allow us to observe the shorter Δ157-fragment. This is because the epitope used for the generation of this antibody is a short sequence

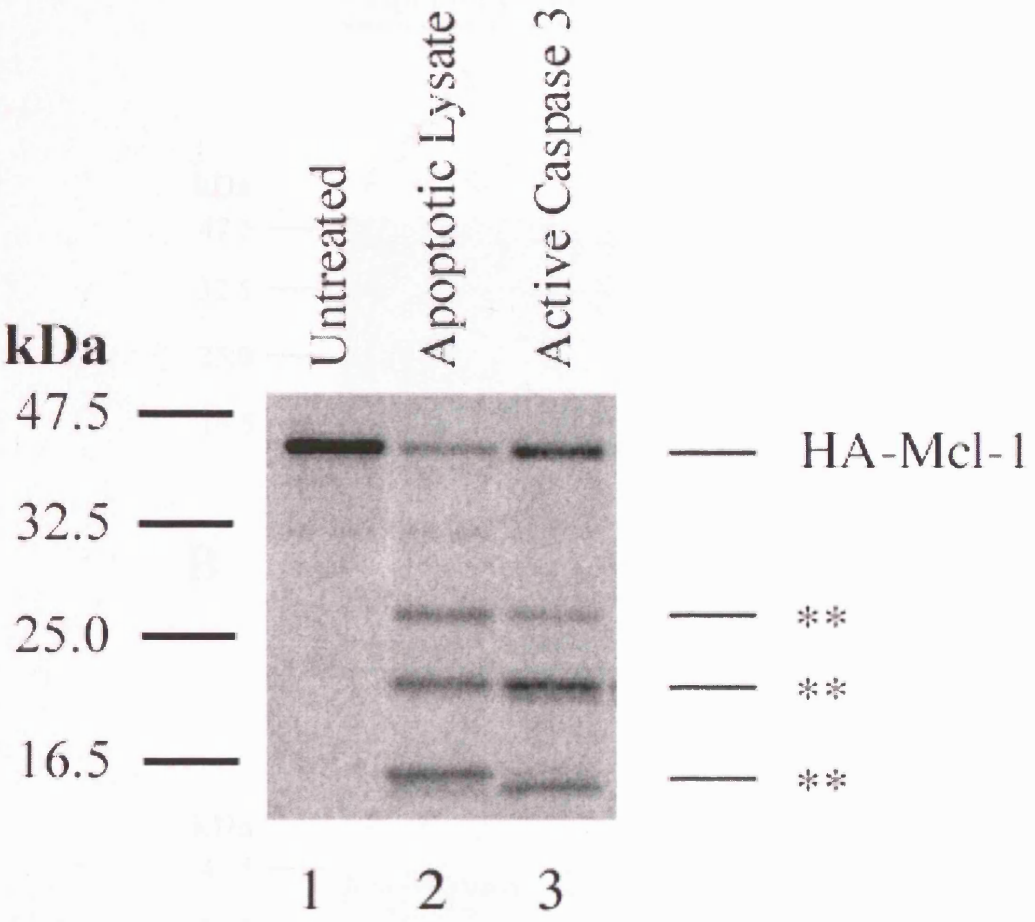


Figure 4-5: The cleavage products of Mcl-1 are generated by caspase cleavage. *In vitro* translated HA-Mcl-1 was left untreated (lane 1) or treated with apoptotic lysate (lane 2) or active caspase-3 (lane 3). The cleavage products are indicated by '**'. Note the lowest band in lane 3 runs slightly faster than the corresponding band in lane 2. This is due to a caspase cleavage site in the HA epitope tag that is more efficiently cleaved by the active caspase-3.

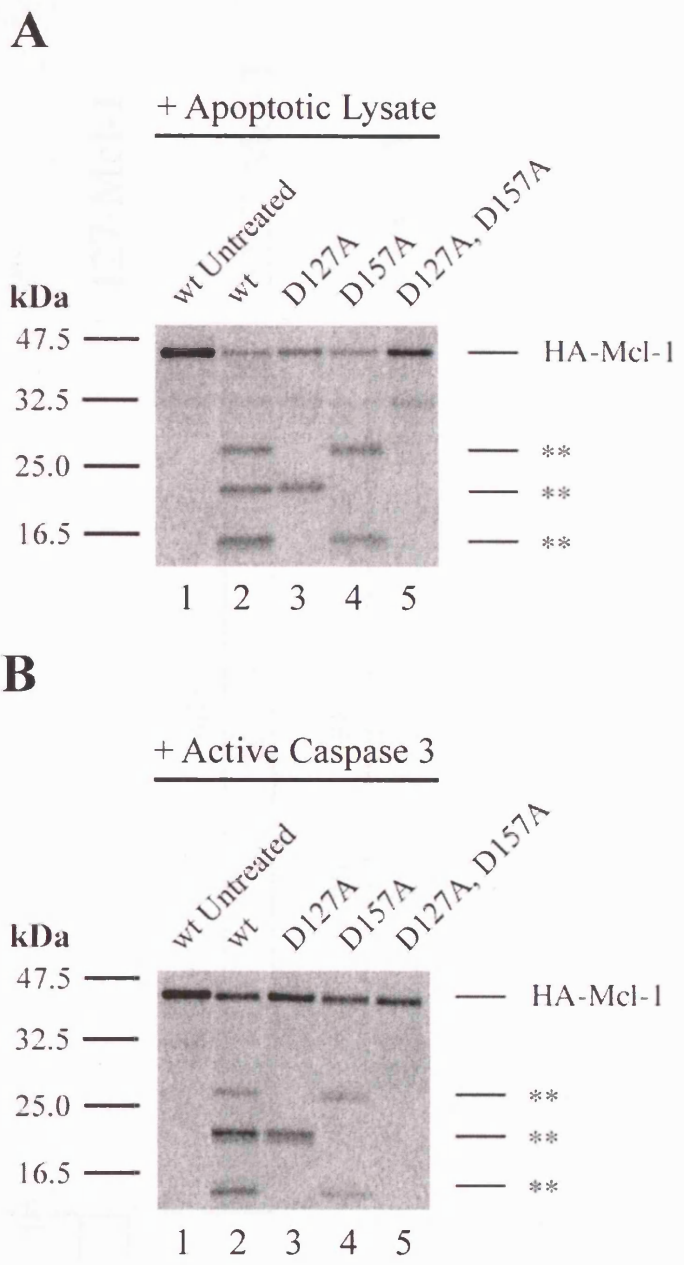


Figure 4-6: Human Mcl-1 is cleaved by caspases at D127 and D157. Aspartic acids at D127 and D157 were mutated to alanine by site-directed mutagenesis. The mutant proteins were treated with apoptotic lysate (A) or active caspase-3 (B). Mutating either D127 (A and B, lane 3) or D157 (A and B, lane 4) alter the cleavage pattern. Cleavage is completely abolished when both resides are mutated (D127A, D157A) (A and B, lane 5). Both apoptotic lysate (A) and active caspase-3 (B) show the same pattern of cleavage.

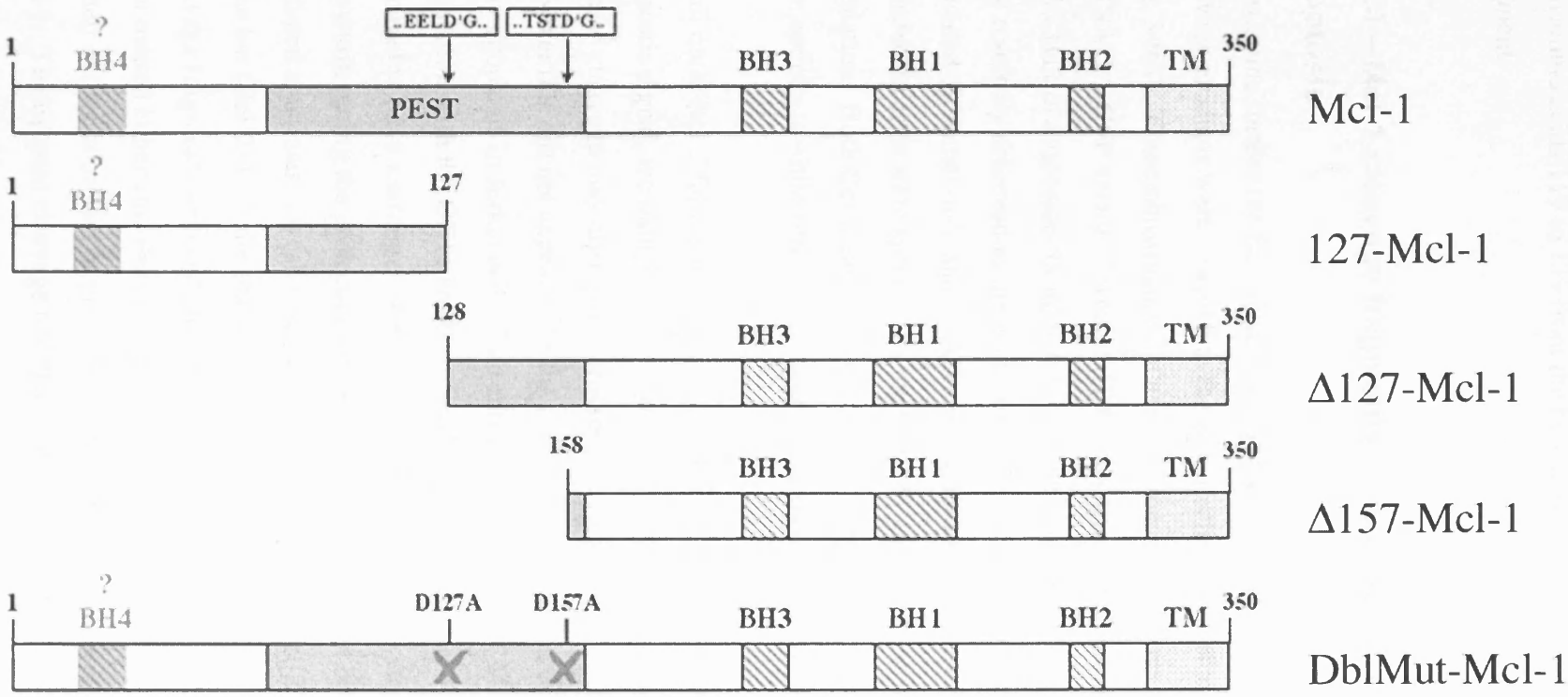


Figure 4-7: Mcl-1 cleavage fragments and double mutant (DblMut) Mcl-1. A schematic outlining the cleavage fragments generated by caspase cleavage of the Mcl-1 protein. These fragments were cloned into expression vectors for further study. The diagram also shows a double mutant (DblMut) Mcl-1 molecule that was also used in these studies.

from amino acids 119 to 139 from the human protein, and is not present in the Δ157 fragment.

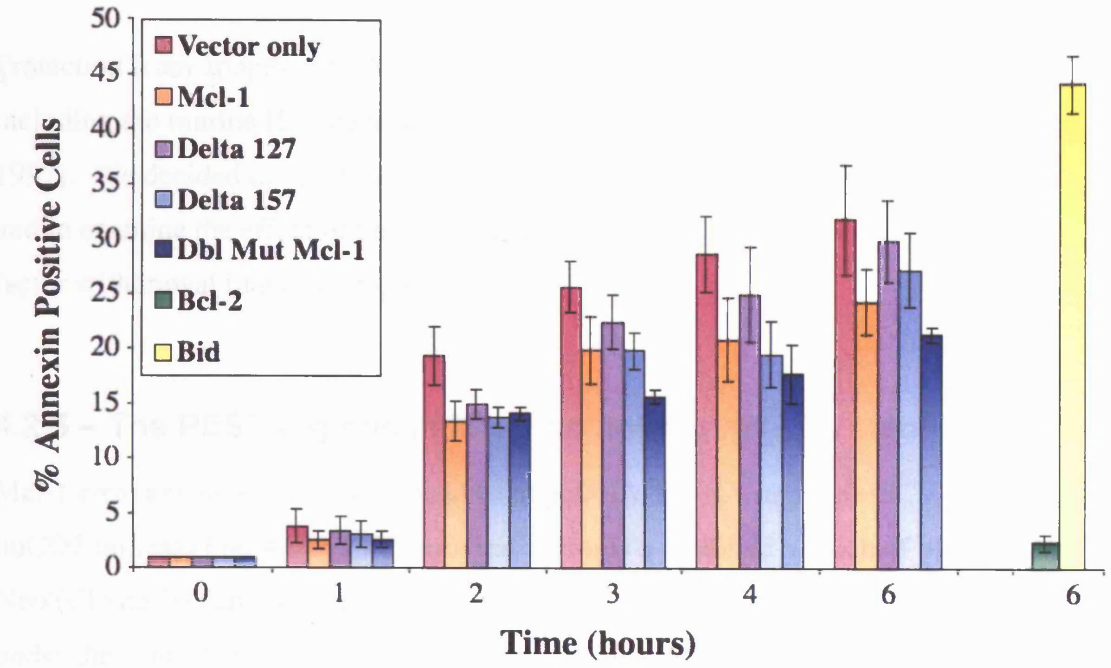
4.2.4 – Mcl-1 cleavage fragments protect Jurkat cells from apoptosis

To examine further the functional consequences of Mcl-1 cleavage each of the cleavage products were cloned into the mammalian expression vector pcDNA3.1-HA (Fig. 3-9A). These constructs were used to transiently co-transfect Jurkat cells with a pcDNA-3.1-GFP vector. Twenty to 24 hours after transfection, cells were treated with CH11 or etoposide for up to 6 hours. Transfection efficiencies of 25 – 30 % were routinely achieved as measured by cells positive for GFP. Apoptosis was measured by Annexin-V and 7AAD staining and analysed by flow cytometry. Transfected cells were specifically analysed by gating on the GFP positive population. Both Bcl-2 and Bid were used as controls. Bcl-2 should protect the cells from apoptosis while Bid should induce a stronger apoptotic phenotype.

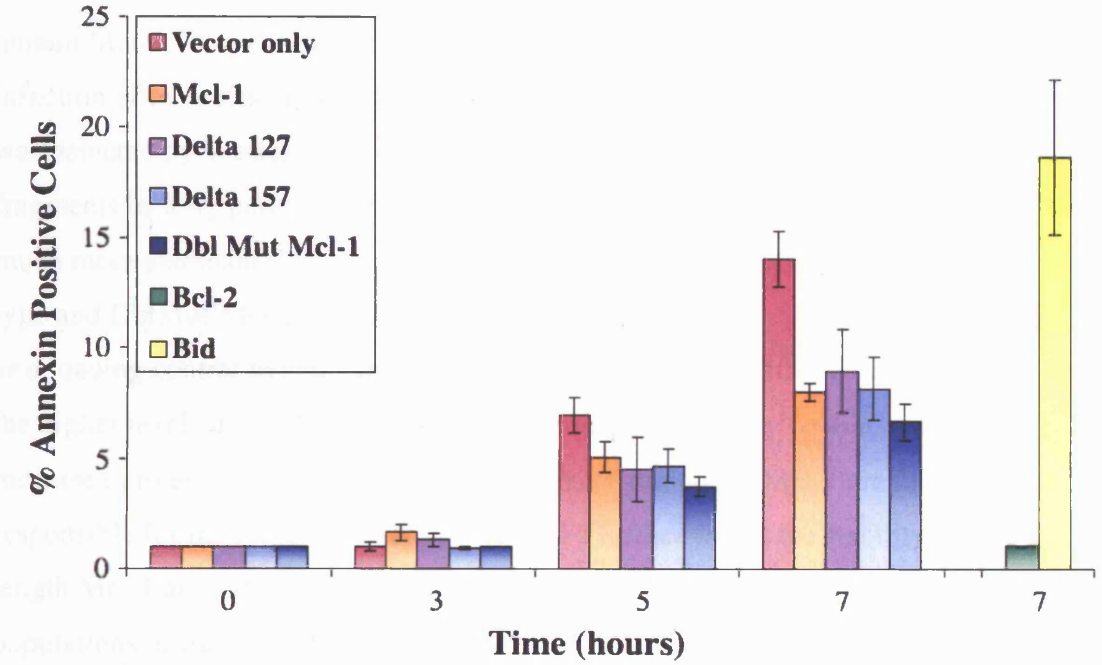
As all other Bcl-2 family members known to be cleaved by caspases result in a pro-apoptotic signal, we thought that the generation of an Mcl-1 'Bax-like' fragment by caspase cleavage may also give a pro-apoptotic phenotype to the cleaved Mcl-1. However this did not appear to be the case. Overexpression of full length Mcl-1 did delay apoptosis in Jurkat cells. The anti-apoptotic effect of Mcl-1 in response to Fas stimulation with the CH11 antibody was not particularly strong (Fig. 4-8A) and Mcl-1 appeared to have a stronger protective effect in response to etoposide (Fig 4-8B). It is also worth noting the protection conferred by Mcl-1 against both Fas and etoposide mediated apoptosis is weak when compared with that of Bcl-2 (Fig. 4-8, compare green bar (Bcl-2) with the orange bar (Mcl-1)). Unexpectedly, the C-terminal cleavage fragments behaved almost exactly as wild-type Mcl-1 when transfected cells were treated either anti-Fas or etoposide, and did not show any significant difference in their ability to protect or promote apoptosis when compared with the wild-type Mcl-1. The caspase cleavage resistant mutant Mcl-1 (DblMut-Mcl-1) was also tested in this system and again gave very similar results as for the wild-type Mcl-1 and the

Figure 4-8: Mcl-1 and its cleavage products delay apoptosis in Jurkat cells. Jurkat cells were co-transfected with Mcl-1 constructs indicated and GFP. Twenty to 24 hours after transfection apoptosis was induced by treatment with 300 ng/ml CH11 anti-Fas (A) or 25 μM etoposide (B). Apoptosis was measured by staining the cells with Annexin-PE and analysing by flow cytometry. By gating on GFP-positive cells transfected populations could be analysed. (A) Mcl-1 and its fragments slightly delay apoptosis induced by CH11 anti-Fas. (B) A greater delay in apoptosis is seen against treatment with etoposide. Note that protection by transfected Bcl-2 is much stronger than that by transfected Mcl-1.

A


Vector only
Mcl-1
Delta 127
Delta 157
Dbl Mut Mcl-1
Bcl-2
Bid
% Annexin Positive Cells
0
5
10
15
20
25
30
35
40
45
50
0
1
2
3
4
6
6
Time (hours)

B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Delta 157
Dbl Mut Mcl-1
Bcl-2
Bid
% Annexin Positive Cells
0
5
10
15
20
25
0
3
5
7
7
Time (hours)

cleavage products. This suggested that at least in Jurkat cells Mcl-1 cleavage by caspases during apoptosis does not significantly alter the function of the Mcl-1 protein.

Protection from apoptosis by Mcl-1 has been characterised in a number of cell types, including the murine IL-3 dependent haematopoetic cell line FDCP-1 (Zhou et al., 1997). We decided to use these cells to further characterise the cleavage fragments and to examine the effect of the individual Mcl-1 cleavage products during growth factor withdrawal induced apoptosis.

4.2.5 – The PEST regions in Mcl-1 do not regulate its stability

Mcl-1 cleavage products were cloned from pcDNA-3.1-HA into a pMSCV-IRES-huCD2-tailless (Fig. 4-9). This retroviral vector is a modified version of pMSCV-Neo (Clontech) that contains a human CD2 gene lacking most of its cytoplasmic tail under the control of an internal ribosome entry site (IRES), thus allowing for detection of transduced cells by the presence of human CD2 on the cell surface. FDCP-1 cells were transduced with constructs containing wild type and double mutant Mcl-1, Mcl-1 cleavage products and the empty vector as a control (Fig. 4-10). Infection efficiencies of 80 – 90 % were routinely achieved and overexpressed protein was detected by Western blot using an anti-HA antibody (Fig. 4-10). We found that fragments lacking part (i.e. Δ127) or all (i.e. Δ157) of the PEST region appeared to be much more abundant (Fig. 4-10, lanes 5 and 6) when compared with full length wild-type and DblMut-Mcl-1 proteins (Fig. 4-10, lanes 3 and 7). Alpha-tubulin was used as a loading control to confirm equal loading in each lane. Initially we thought that the higher levels of the Δ127 and Δ157 cleavage products may be as a result of increased protein stability, especially as the PEST regions in Mcl-1 are thought to be responsible for the rapid turnover of Mcl-1. To further assess the stability of full length Mcl-1 and the Δ127-Mcl-1 and Δ157-Mcl-1 cleavage fragments the mixed populations of transduced cells were treated with the translation inhibitor cyclohexamide (CHX) for 0, 10, 20, 30, 60, 90 or 120 minutes (Fig. 4-11). The Western blots in Figure 4-11 show that Mcl-1 and the two cleavage fragments are

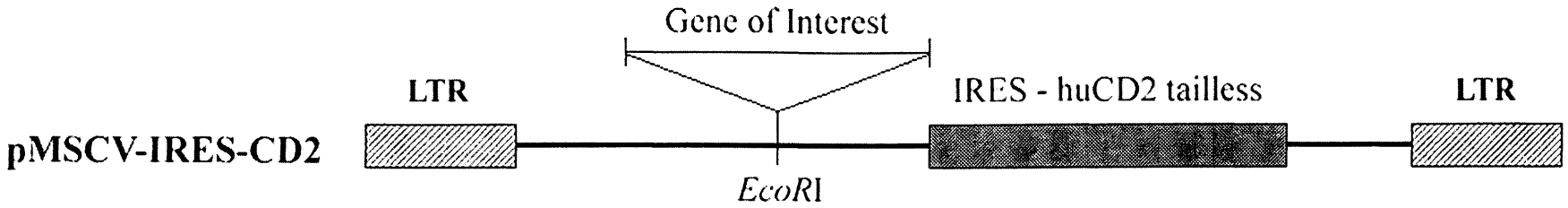


Figure 4-9: pMSCV-IRES-huCD2 tailless. The retroviral vector pMSCV-IRES-huCD2 tailless was used for retroviral infections of FDCP-1 cells. The vector is a modified version of the pMSCV-Neo vector that is commercially available from Clontech. The neomycin gene in pMSCV-Neo has been replaced with an internal ribosome entry sequence (IRES) that is followed by a human CD2 construct that lacks most of its cytoplasmic tail. This allows for easy detection of infected cells by analysing for CD2 levels on the cell surface.

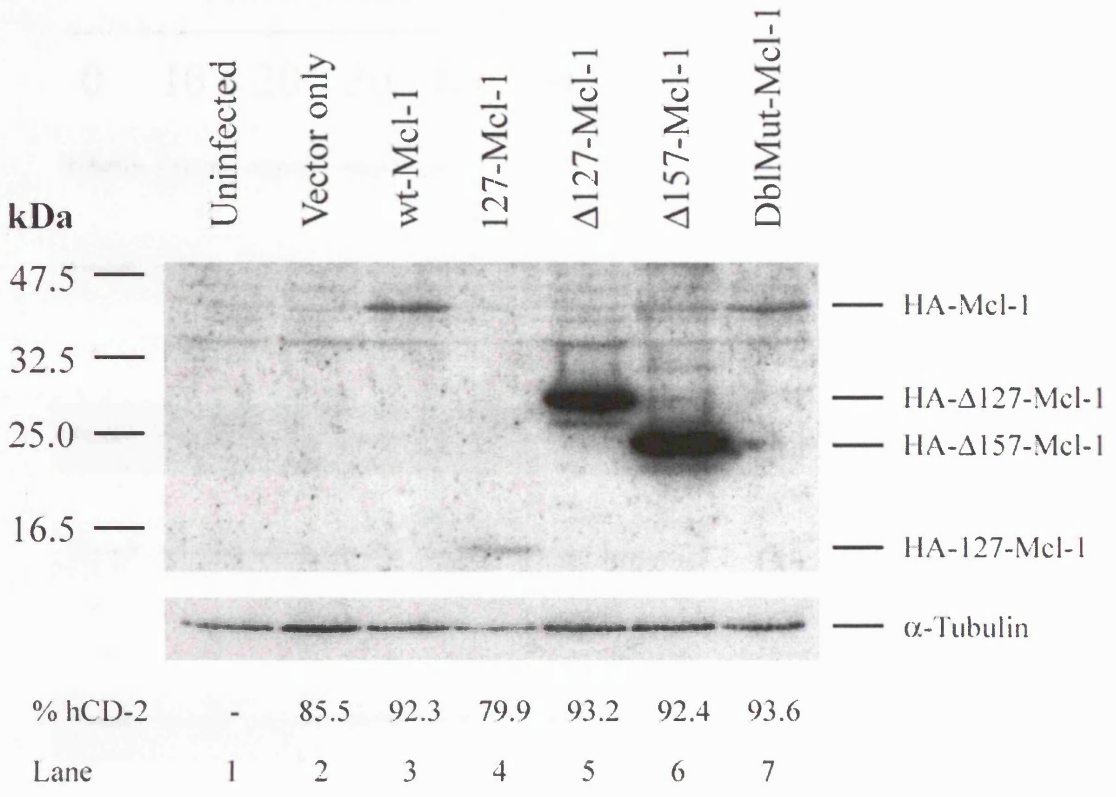


Figure 4-10: Overexpression of Mcl-1 constructs in transduced FDCP-1 cells. FDCP-1 cells were retrovirally infected with pMSCV-IRES-huCD2 constructs containing Mcl-1 and its cleavage products. CD2 on the cell surface was measured by flow cytometery and the percentage of infected cells expressing CD2 on their surface is shown below each lane. A western blot of total cell lysate was blotted using an anti-HA antibody to detect expression of the exogenous protein. The blot was stripped and probed using an anti-α-tubulin antibody to check for equal loading. Much more protein was present for the Δ127- (lane 5) and Δ157-Mcl-1 (lane 6) constructs than for full length Mcl-1 proteins (lanes 3 and 7). This cannot be as a result of equal loading of protein or higher infection efficiency.

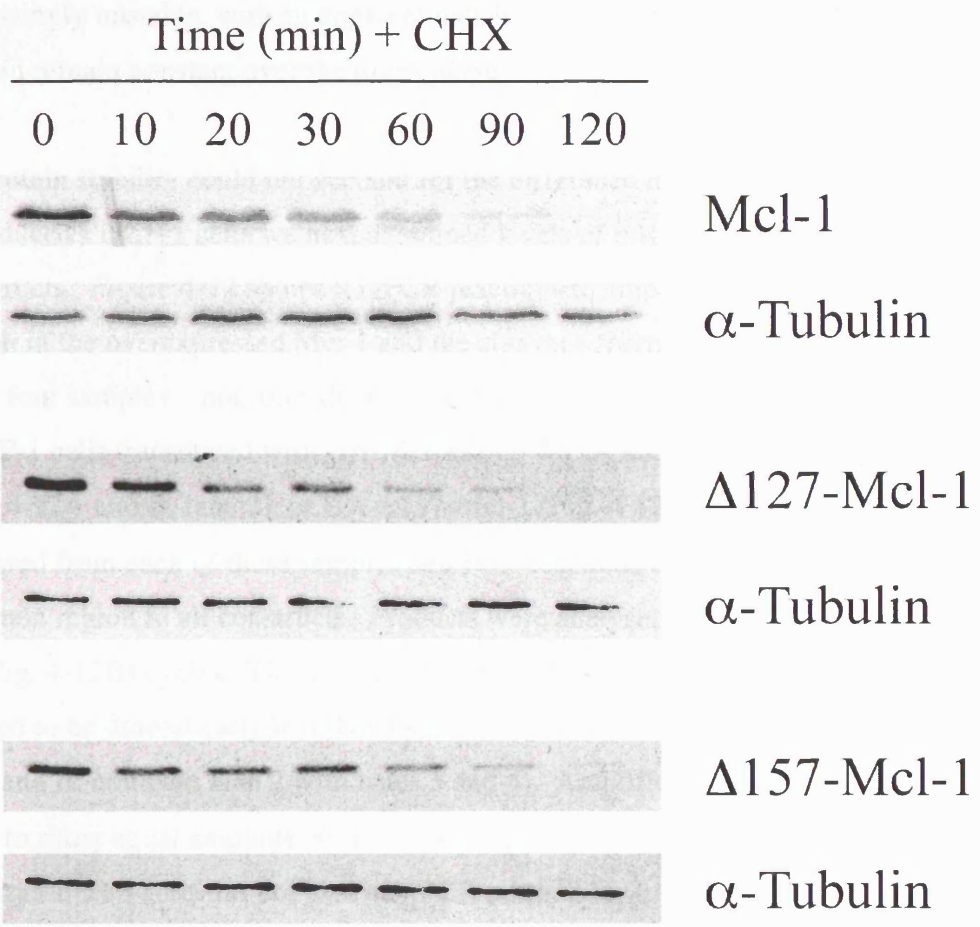


Figure 4-11: PEST regions of Mcl-1 are not responsible for protein stability in FDCP-1 cells. FDCP-1 cells stably transduced with Mcl-1 constructs expressing full length HA-Mcl-1, HA-Δ127-Mcl-1 or HA-Δ157-Mcl-1 were incubated with 30 μg/ml cyclohexamide (CHX) for the times indicated to prevent translation of the exogenous proteins. Western blots on total cell lysate of samples were probed with an anti-Mcl-1 antibody (HA-Mcl-1) or anti-HA antibody (for HA-Δ127-Mcl-1 and HA-Δ157-Mcl-1) and degradation of the protein observed over the time course indicated. The blots were also probed with an anti-α-tubulin antibody to check for equal loading. There appears to be no degradation of α-tubulin over this time course.
there to be no real difference in the rate of degradation between the protein products expressed from the constructs.

surprisingly unstable, with an apparent half-life of < 30 min, while the levels of α-tubulin remain constant over the time course.

As protein stability could not account for the difference in protein levels in the transduced FDCP-1 cells we next examined levels of mRNA for each of the constructs. Figure 4-12 shows RT-PCR reactions to amplify a common C-terminal region in the overexpressed Mcl-1 and the cleavage fragments. RNA was prepared from four samples – non-transduced FDCP-1 cells (Fig. 4-12A and B, lane 1) and FDCP-1 cells transduced with HA-Mcl-1 (Fig. 4-12A and B, lane 2), HA-Δ127-Mcl-1 (Fig. 4-12A and B, lane 3), or HA-Δ157-Mcl-1 (Fig. 4-12A and B, lane4). cDNA was prepared from each of these samples and PCR reactions carried out to amplify a common region to all constructs. Products were analysed after 27 (Fig. 4-12A) and 30 (Fig. 4-12B) cycles. The overall level of mRNA present for full length Mcl-1 proved to be dramatically less than that for either Δ127-Mcl-1 or Δ157-Mcl-1 (Fig. 4-12A and B, compare lane 2 with lanes 3 and 4). Amplification of β-actin by PCR was used to show equal amounts of cDNA were used for PCR reactions. As expected, non-transduced cells did not give any PCR product for exogenous Mcl-1 expression (Fig. 4-12A and B, lane 1). This large difference in the amount of mRNA present in the cells explains the difference in protein levels seen in Figure 4-10.

4.2.6 – Δ157 fragment of Mcl-1 can still protect from apoptosis

We carried out apoptosis assays using the transduced FDCP-1 cells to investigate if the Mcl-1 cleavage products would promote apoptosis in this system. Apoptosis was induced by withdrawing growth factors withdrawal for a period of five days and monitored by measuring the percentage of sub-G1 cells each day using a flow cytometer. Contrary to previously published data (Zhou et al., 1997) Mcl-1 did not appear to delay apoptosis in these cells when compared to empty vector infected cells (Fig. 4-13A and B). Interestingly neither the Δ127- nor DblMut-Mcl-1 fragments appeared to have any pro-apoptotic function (Fig. 4-13A). In fact, only the Δ157 fragment showed a significant difference when compared with empty vector control infected cells (Fig. 4-13B). The protection by Δ157-Mcl-1 appeared to be statistically significant at all time points with a $p = 0.0036$ at Day 5 (Fig. 4-13B).

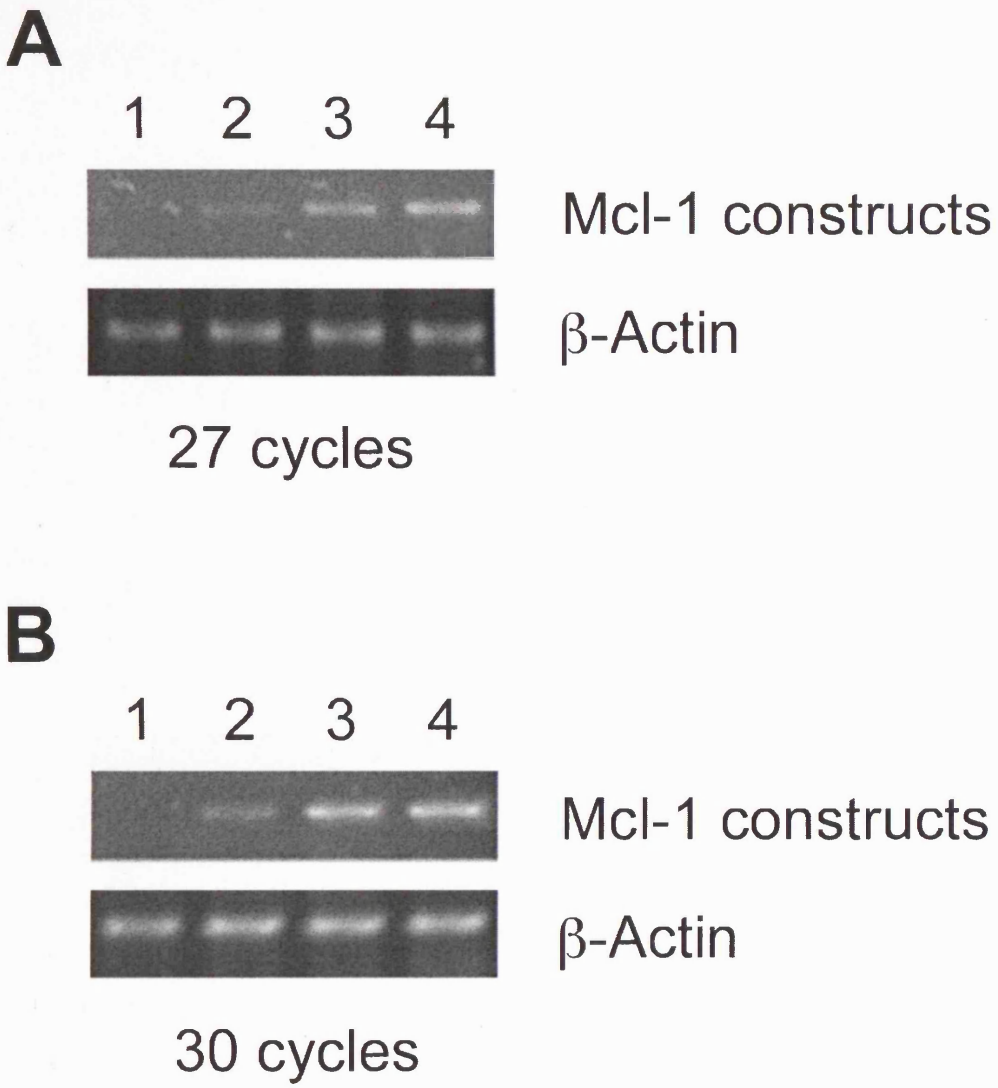
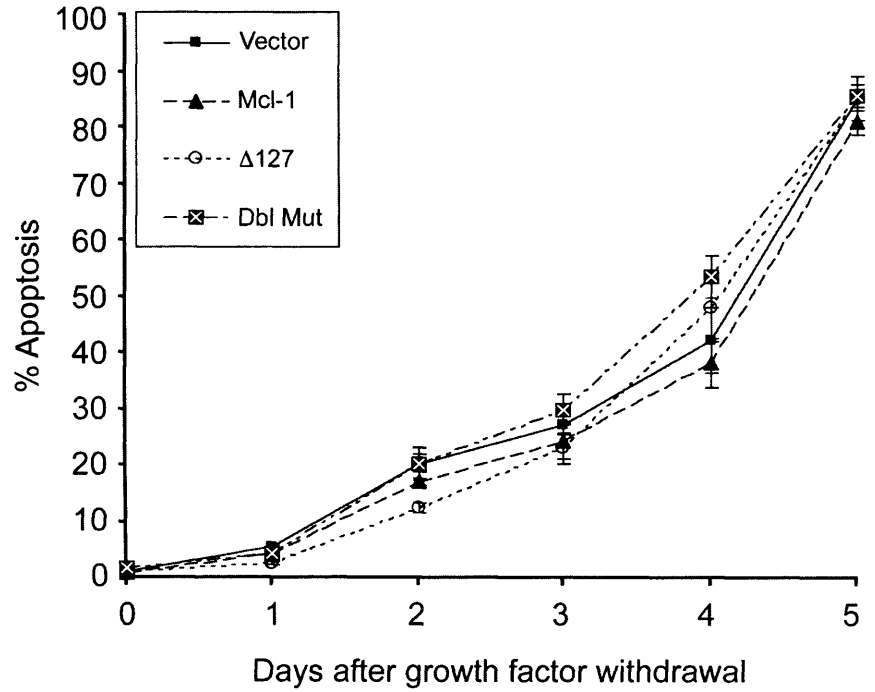


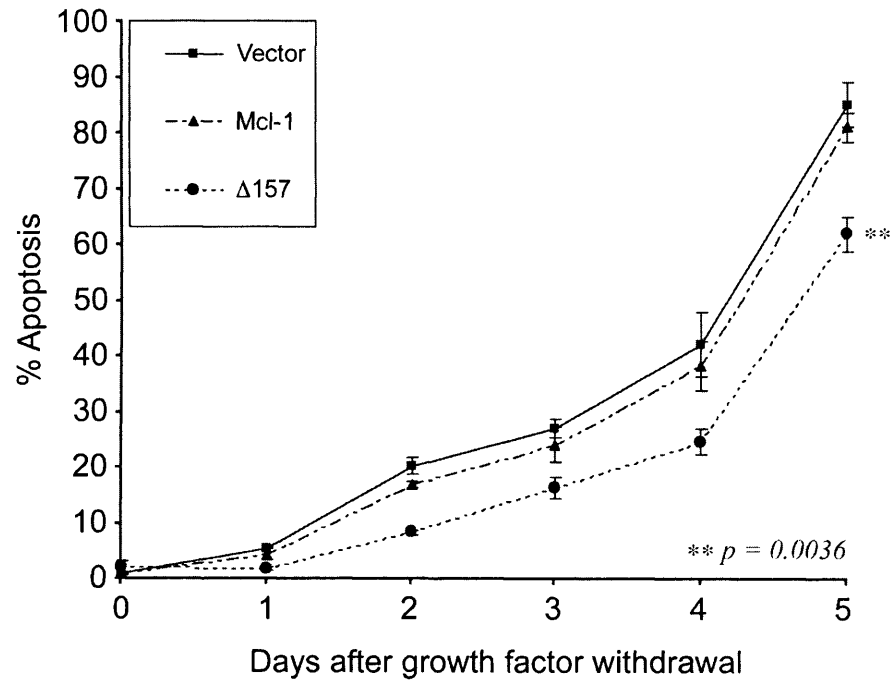
Figure 4-12: Relative mRNA levels of Mcl-1, Δ127-Mcl-1 and Δ157-Mcl-1 as determined by RT-PCR. cDNA was generated from FDCP-1 cells that had not been transduced (lane 1) or transduced with HA-Mcl-1 (lane 2), HA- Δ127-Mcl-1 (lane 3) or HA-Δ157-Mcl-1 (lane 4). PCR was carried out on each cDNA sample to amplify a region common to all constructs. Aliquots were taken from tubes after 27 cycles (A) and 30 cycles (B) and clearly show that the levels of Mcl-1 mRNA are lower than those of HA- Δ127-Mcl-1 and HA-Δ157-Mcl-1. PCR of β-actin was used to show that equal amounts of RNA had been used for each lane.

Figure 4-13: Δ157-Mcl-1 can protect FDCP-1 cells from apoptosis induced by growth factor withdrawal. Apoptosis of FDCP-1 cells that had been stably transduced with Mcl-1 was induced by growth factor withdrawal. Neither Mcl-1, DblMut-Mcl-1, nor Δ127- Mcl-1 protected from apoptosis in this system (A). Neither did they promote apoptosis. However Δ157-Mcl-1 did show protection from growth factor withdrawal mediated apoptosis.

A


Vector
Mcl-1
Δ127
Dbl Mut
% Apoptosis
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
Days after growth factor withdrawal

B


Vector
Mcl-1
Δ157
% Apoptosis
100
90
80
70
60
50
40
30
20
10
0
**
** p = 0.0036
0
1
2
3
4
5
Days after growth factor withdrawal

Again, this fragment shows no pro-apoptotic potential but appears to retain the anti-apoptotic function of full-length Mcl-1, and can significantly delay apoptosis in FDCP-1 cells in response to withdrawal of growth factor.

4.3 – Discussion

There are a number of Bcl-2 family members that are now known to be cleaved by caspases. The best characterised of these is Bid, the BH-3 only protein. Bid is a relatively inactive protein that exists as a p22 form within the cell. Cleavage of Bid by caspase-8 results in the generation of the potently apoptotic p15 tBid fragment (Li et al., 1998; Luo et al., 1998). As outlined already this fragment translocates to the mitochondria where it plays an important role in apoptosis and has the ability to amplify apoptotic signals through stimulating the release of cytochrome-c from the mitochondria and subsequent activation of caspase-9. Bad and Bim are other BH-3 only proteins that are cleaved by caspases (Chen and Zhou, 2004; Condorelli et al., 2001). Bad is normally regulated through phosphorylation and sequestered by 14-4-3 proteins in the cytoplasm (Datta et al., 2000; Zha et al., 1996). However it is known to be cleaved by caspases, and this cleavage results in a significant increase in its pro-apoptotic activity (Condorelli et al., 2001). Similarly, Bim is a sequestered to the microtubule-associated dynein motor complex within the cell, after release from the complex during early apoptosis Bim_{EL} can be cleaved by caspases induces a strong pro-apoptotic effect(Chen and Zhou, 2004). Cleavage of these proteins results in an increase to their already pro-apoptotic potential. The anti-apoptotic proteins Bcl-2 and Bcl-X_L are also known to be cleaved by caspases (Cheng et al., 1997; Fujita et al., 1998). Cleavage of these proteins results in the removal of their BH-4 domain. This generates a 'Bax-like' fragment, containing BH-3, BH-1 and BH-2 domains. The result is that these previously anti-apoptotic proteins become quite potently pro-apoptotic.

In a similar fashion, we have shown here that Mcl-1 is cleaved by caspases to become a 'Bax-like' protein, lacking a BH-4 homology domain. However our experiments in both Jurkat-T cells and FDCP-1 cells show that overexpression of the cleavage fragments does not show any pro-apoptotic potential and that these fragments appear to retain an anti-apoptotic function. It is interesting to note that in these overexpression studies the cleavage fragments were much more abundant relative to

full-length Mcl-1 fragments, suggesting that any anti-apoptotic potential these fragments may have is not as strong as the full length protein.

In the case of Jurkat-T cells, overexpression of the cleavage fragments showed a similar level of protection to that of the full-length Mcl-1 for both Fas and etoposide mediated apoptosis. In the case of Fas induced apoptosis there is a trend in which expression of these fragments may have the ability to delay apoptosis, although this is not a significant delay in apoptosis; while all fragments offer a better protection from etoposide induced apoptosis. This ability of Mcl-1 to protect better from etoposide induced apoptosis is consistent with the published role of Mcl-1 in apoptosis mediated through DNA damage (Cuconati et al., 2003; Nijhawan et al., 2003). What is interesting is that although the protein levels of the Δ127-Mcl-1 and Δ157-Mcl-1 are much higher than full-length Mcl-1 they do not appear to show an enhanced protective effect. This suggests that these fragments may not be as anti-apoptotic as full-length Mcl-1.

FDCP-1 cells were also used to examine protection by Mcl-1. These cells were stably transduced using a retroviral gene transfer system. Expression of both CD2-tailless and HA-tagged Mcl-1 constructs was well maintained over a period of weeks. This suggested that the overexpression of Mcl-1 caspase cleavage fragments did not have any seriously damaging effects on cell growth or proliferation. It has been previously published that wild-type full-length Mcl-1 can protect these cells from growth factor withdrawal induced apoptosis. However we were unable to detect any protective effect of Mcl-1 when compared with vector only control cells. It is unclear why this may be the case. FDCP-1 cells are murine myeloid progenitor cells that were originally generated from a population of murine bone-marrow cells, grown in the presence of IL-3 to generate IL-3 dependent cells (Dexter et al., 1980). There are a number of different populations of these cells - FDCP-Mix, FDCP-1 and FDCP-2 cells, which were generated from the original IL-3 dependent population but have quite different characteristics. It is thought that many of these clones have diverged over the years resulting in a number of groups calling their cell lines FDCP-Mix, FDCP-1 or FDCP-2 cells when they may not be representative of the original cell populations that were characterised (Spooncer et al., 1993). We purchased our

FDCP-1 cells directly from ATCC and they may not be exactly the same as those cells used in previously published data showing protection by Mcl-1. However, the Δ157-Mcl-1 fragment did delay apoptosis in these cells. This protection was consistent over a number of experiments and was statistically significant at all time points when compared with control cells. The reason for this increased protection may be in part as a result of the higher levels of Δ157-Mcl-1 protein. If this were the only reason for the increased protection of Δ157-Mcl-1 then Δ127-Mcl-1 should also offer increased protection as there is significantly more of this fragment compared with full length Mcl-1. This does not appear to be the case however.

Mcl-1 is known to have a rapid turnover within the cell and this has been thought to be as a result of two PEST regions within the protein. When we overexpressed the Δ127-Mcl-1 and Δ157-Mcl-1 fragments in both Jurkat-T cells and FDCP-1 cells, we initially assumed this to be as a result of the removal of the PEST regions from the protein. Investigation into Mcl-1 stability in FDCP-1 cells however showed that this was not the case, and that the real reason for lack of full-length Mcl-1 in the cells was lower mRNA levels. In fact our results showed that the stability of the Mcl-1 protein did not appear to be affected by removal of the PEST regions in FDCP-1s. This confirms findings for Mcl-1 overexpressed in U937 cells (Akgul et al., 2000) and suggests that there is a region in the C-terminus of Mcl-1 that is responsible for the short half-life of Mcl-1. It also brings into question the functional significance of this large N-terminal region if it is not responsible for rapid turnover of Mcl-1 within the cell.

5 - Discussion

The initial aim of this project was to identify novel interaction partners for the pro-apoptotic BH3-only protein tBid. We identified two novel interaction partners, EID-1 and Mcl-1. During the course of this work we also observed that Mcl-1 was cleaved during apoptosis in the Jurkat T cell line. Consequently we characterised the cleavage of the Mcl-1 protein and attempted to determine the functional effects of the cleavage products.

There is little known about EID-1 and no evidence to suggest that EID-1 may play a role in apoptosis. Its published role relates to controlling gene expression in combination with histone deacetylases (HDACs) and histone acetyltransferases (HATs) (Bavner et al., 2002; MacLellan et al., 2000; Miyake et al., 2000). EID-1 is down regulated during differentiation of myoblasts and the haematopoetic cell line U937 and overexpression of the protein is able to block muscle specific gene expression and differentiation of C2C12 myoblasts (MacLellan et al., 2000; Miyake et al., 2000). Exogenous EID-1 appears to be localised to both the nucleus and cytoplasm (Bavner et al., 2002). Assuming that endogenous EID-1 has the same localisation pattern, it is possible that it may be able to interact with tBid during apoptosis. As well as the ability of EID-1 to interact with tBid, it may also interact with other Bcl-2 family members. Without evidence of a role for EID-1 during apoptosis it is difficult to see how this would have a functional significance for cell survival. For this reason we felt that that the interaction between Mcl-1 and tBid was a more relevant interaction and concentrated on experiments to determine the functional significance of this interaction.

Mcl-1 is an anti-apoptotic protein with a clear role in development and differentiation. As an anti-apoptotic Bcl-2 family member it is likely that Mcl-1 and tBid interact with each other *in vivo*. The next step in evaluating the interaction between these two Bcl-2 proteins is to show that this interaction occurs *in vivo* and to show that it has a functional significance. Co-immunoprecipitation of the proteins from cell lysates

should confirm that Mcl-1 interacts with tBid. This, along with the yeast-two hybrid and GST data, would be very strong evidence for the interaction occurring *in vivo*. This interaction is most likely to occur via the BH domains of both proteins. Based on the yeast-two hybrid data presented in Chapter 3, we know that it is the C terminal region of Mcl-1 that is important for interaction with tBid and it is this region in Mcl-1 that contains the BH1, -2, and -3 domains. Mutation of these BH-domains should confirm if these domains are responsible for mediating the interaction between the proteins.

It has previously been shown that tBid can induce the release of cytochrome *c* from isolated mitochondria. We would predict that this tBid mediated cytochrome *c* release could be inhibited by the addition of exogenous Mcl-1. Any inhibition of cytochrome c release by Mcl-1 would be due to the ability of Mcl-1 to interact directly with tBid. In this situation Mcl-1 would act by sequestering tBid and preventing it from activating Bax or Bak (see Fig. 1-4). Thus by sequestering the *'activating'* tBid, this would prevent oligomerisation of Bak and Bax. This can be readily examined using isolated mitochondria *in vitro* and inducing cytochrome *c* release by addition of exogenous tBid in the presence of Mcl-1. By generating Mcl-1 overexpressing stable cell lines we can examine the protection offered by Mcl-1 *in vivo* in response to Bid mediated apoptosis.

It is interesting that in-depth analysis of the Bid null mice showed myeloid expansion, with an increased absolute neutrophil count (Zinkel et al., 2003). This myeloid hyperplasia leads to pre-leukaemic phenotype that progresses to a fatal clonal disorder that resembles human chronic myelomonocytic leukaemia (CMML) (Zinkel et al., 2003). The authors of the study say that the myelomonocyte lineage of these *Bid*-deficient mice "is singularly dependent on Bid for physiologic cell death." The Mcl-1 protein also plays a vital role in survival of haematopoietic cells and myeloid cells in particular. *In vitro* culturing of cells from Mcl-1 transgenic mice show enhanced survival of haematopoietic cells in general with immortalisation of cells in the myeloid lineage (Zhou et al., 1998). In light of the results shown here, the fact that a lack of Bid or excess of Mcl-1 can provide cells of the myeloid lineage with a survival advantage suggests that these proteins co-operate to ensure proper

homeostasis and maintenance of the myeloid lineage. Further study is required to determine the importance of Bid cleavage for its role in maintenance of myeloid homeostasis, as well as to examine whether the balance between Bid and Mcl-1 and the ability of these proteins to interact can influence normal myeloid homeostasis.

After identifying Mcl-1 as a novel interaction partner for tBid, we subsequently found it to be cleaved during apoptosis of Jurkat T cells. There have been a number of Bcl-2 family members that have been identified as targets for caspases (Chen and Zhou, 2004; Cheng et al., 1997; Condorelli et al., 2001; Fujita et al., 1998; Li et al., 1998; Luo et al., 1998). In all cases to date, apart from Mcl-1, cleavage of Bcl-2 proteins by caspases results in the generation of a more potently apoptotic protein. We initially expected that this would also be case for Mcl-1. However experiments in both Jurkat T cells (where the cleavage was initially observed) and the IL-3 dependant FDCP-1 cell line gave no indication that the cleavage products were pro-apoptotic. In fact, it is possible that one of the fragments may potentially retain some anti-apoptotic activity. Whether cleavage of Mcl-1 by caspases generates apoptotic fragments, neutralises the anti-apoptotic potential of the fragment, or has any effect on the function of Mcl-1 may depend on the context in which such fragments are generated.

A number of papers have detailed the roles of caspase proteins outside of apoptosis. Indeed the first caspase identified (caspase-1) was originally identified as an enzyme involved in the biosynthesis of interleukin-1-β (Thornberry, 1994). Since then other caspases have been implicated with roles in the maturation of other cytokines as well as being identified as having roles in differentiation (Abraham and Shaham, 2004).

This potential role of caspases in differentiation could have important implications for a role of Mcl-1 in differentiation. Caspase-3 has been shown to be activated in U937 cells in response to differentiation with PMA (Pandey et al., 2000) and also to be activated during epidermal keratinocyte and erythroid differentiation (Weil et al., 1999; Zermati et al., 2001). As caspase cleavage of Mcl-1 does not appear to alter its anti-apoptotic function dramatically, potential caspase activation during differentiation and possible cleavage of Mcl-1 during these events would make Mcl-1

a much better protector under these circumstances than other anti-apoptotic Bcl-2 proteins.

Cleavage of Mcl-1 can generates two main fragments, a larger C terminal fragment and a smaller N terminal fragment. The small fragment generated contains the PEST regions within the Mcl-1 protein. We speculated that this would generate more stable C terminal fragments, however this was not the case. In fact, the PEST regions do not appear to influence the stability of the protein and fragments lacking the PEST domains have a half-life similar to the full-length protein. This implies that the N terminal region of Mcl-1 may have a function that has yet to be determined.

This also raises an interesting question for the role of Mcl-1's BH-4 domain contained at the N terminus of the protein. Might its N-terminal region, containing the BH-4 domain, influence differentiation and development, particularly as the PEST region does not appear to be responsible for the short half-life of Mcl-1? If Mcl-1 is cleaved during differentiation, this would release this region from the rest of the protein leaving it free to act elsewhere within the cell.

Interestingly the BH4 domains of other Bcl-2 family members have been implicated in determining cell fate during differentiation. It has been known for some time that Bcl-X_L plays a role in erythroid differentiation. Part of this role may be independent of its anti-apoptotic function (Hafid-Medheb et al., 2003). In experiments with differentiating FDCP-Mix cells, Bcl-X_L promotes differentiation to an erythroid lineage, while Bcl-2 promotes differentiation to a monocyte/macrophage lineage (Haughn et al., 2003). When the BH-4 domain of Bcl-2 was replaced with that of Bcl-X_L, cells containing the modified Bcl-2 were no longer able to differentiate to monocytes/macrophages and instead gave erythroid cells. FDCP-Mix cells containing Bcl-2 showed lower levels of Raf-1, a protein known to influence erythropoiesis, while using the modified Bcl-2 restored levels of Raf-1 as seen in FDCP-Mix cells expressing Bcl-X_L (Haughn et al., 2003). Perhaps the N terminal region of Mcl-1 may have similar properties that have yet to be identified.

This thesis describes a number of interesting observations relating to the Bcl-2 family proteins Bid and Mcl-1. Based on the work presented here and data available in the literature we would hypothesise that these proteins have an important influence on each other that has not yet been acknowledged. Further work to characterise the interaction between tBid and Mcl-1 to determine its significance, as well as caspase cleavage of Mcl-1, may help elucidate the molecular mechanisms by which they influence apoptosis and clarify their physiological roles.

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Appendix I

Primers used for PCR reactions:

3´BaxΔ21-*Cla* I (5´-CC**ATCGAT**TCACTGCCATGTGGG-3´)

3´CDC37-*Bam*H I (5´-CG**GGATCC**TCACACACTGACATCCTTCTCATCGC-3´)

3´GST-Bid-*Xho* I (5´-CCG**CTCGAG**TCAGTCCATCCCATTTCTGGC-3´)

3´GST-Mcl-1-*Eco*R I (5´-CCG**GAATTC**CTATCTTATTAGATATGCC-3´)

3´GST-EID-1- *Eco*R I (5´-CCG**GAATTC**CTACTCTCTATCAATAATC-3´)

3´hBid-*Bam*H I (5´-CG**GGATCC**TCAGTCCATCCCATTTCTGGC-3´)

3´hEID-1-*Bam*H I (3´-CG**GGATCC**CTACTCTCTATCAATAATGTC-3´)

3´hNM23B-*Bam*H I (5´-CG**GGATCC**TTATTCATAGACCCAGTC-3´)

3´mBid-*Bam*H I (5´-CG**GGATCC**TCAGTCCATCTCGTTTCTAACCAAG-3´)

3´Mcl-1-*Bam*H I (5´-GC**GGATCC**TCAGTCGCTGAAAACATGG-3´)

3´Mcl-127-R (5'-CGC**GGATCC**CTAGTCCAGCTCCTCTTCGG-3')

5´BaxΔ21-*Eco*R I (5´-CCG**GAATTC**ATGGACGGGTCCGG-3´)

5´GST-Bid-*Eco*R I (5´-CCG**GAATTC**CCATGGACTGTGAGGTCAACAACG-3´)

5´GST-EID-1- *Bam*H I (5´-CGC**GGATCC**CCAATGTCGGAAATGGC-3´)

5´GST-Mcl-1-*Bam*H I (5´-
CGC**GGATCC**CCAATGTTTGGCCTCAAAAGAAACG-3´)

5´GST-tBid-*Eco*R I (5´-CCG**GAATTC**CCGGCAACCGCAGCAGCCAC-3´)

5´hBid-*Eco*R I (5´-CG**GAATTC**ATGGACTGTGAGGTCAACAACG-3´)

5´hCDC37-*Eco*R I (5´-CG**GAATTC**ATGGTGGACTACAGCCTGTGG-3´)

5´hEID-1-*Eco*R I (5´-CG**GAATTC**ATGTCGGAAATGGCTGAGTTGTCC-3´)

5´hNM23B-*Eco*R I (5´-CG**GAATTC**ATGGCCAACCTGGAGCGC-3´

5´htBid-*Eco*R I (5´-CG**GAATTC**ACCATGGGCAACCGCAGCAGC-3´)

5´Mcl-Δ127-F (5′-CCG**GAATTC**GGGTACGAGCCGGAGCCTCTCGG-3′)

5´Mcl-Δ157-F (5′-CCG**GAATTC**GGGTCACTACCCTCGACGC-3′)

5´Mcl-1-*Eco*R I (5´-CG**GAATTC**ATGTTGGCCTC-3´)

5´mtBid-*Eco*R I (5´-CCG**GAATTC**GGCAGCCAGGCCAGC-3´)

Actin F3 (5′-GGCATCCACGAAACTACCTTCAA-3′)

Actin B3 (5′-AACCGACTGCTGTCACCTTCAC-3′)

D127A-F (5′-CATGTCGCCCGAAGAGGAGCTG**G**CCGGGTACGAGC-3′)

D127A-R (5′-CAGCTCCTCTTAGGGCGACATGATGGCGTCAGC-3′)

D157A-F (5′-GGTAATAACACCAGTACG**G**CCGGGTCACTACCC-3′)

D157A-R (5′-CGTACTGGTGTTATTACCAGATTCCCCGACC-3′)

Mcl-1-3F (5′-TGCAGCGCAACCACGAGACCGG-3′)

pcDNA3.1-R (5′-AAAGCTTGGTACCGAGCTCGG-3′)

Appendix II

Yeast two-hybrid filters stained with Z-Buffer to identify interaction partners for tBid

Plate 1

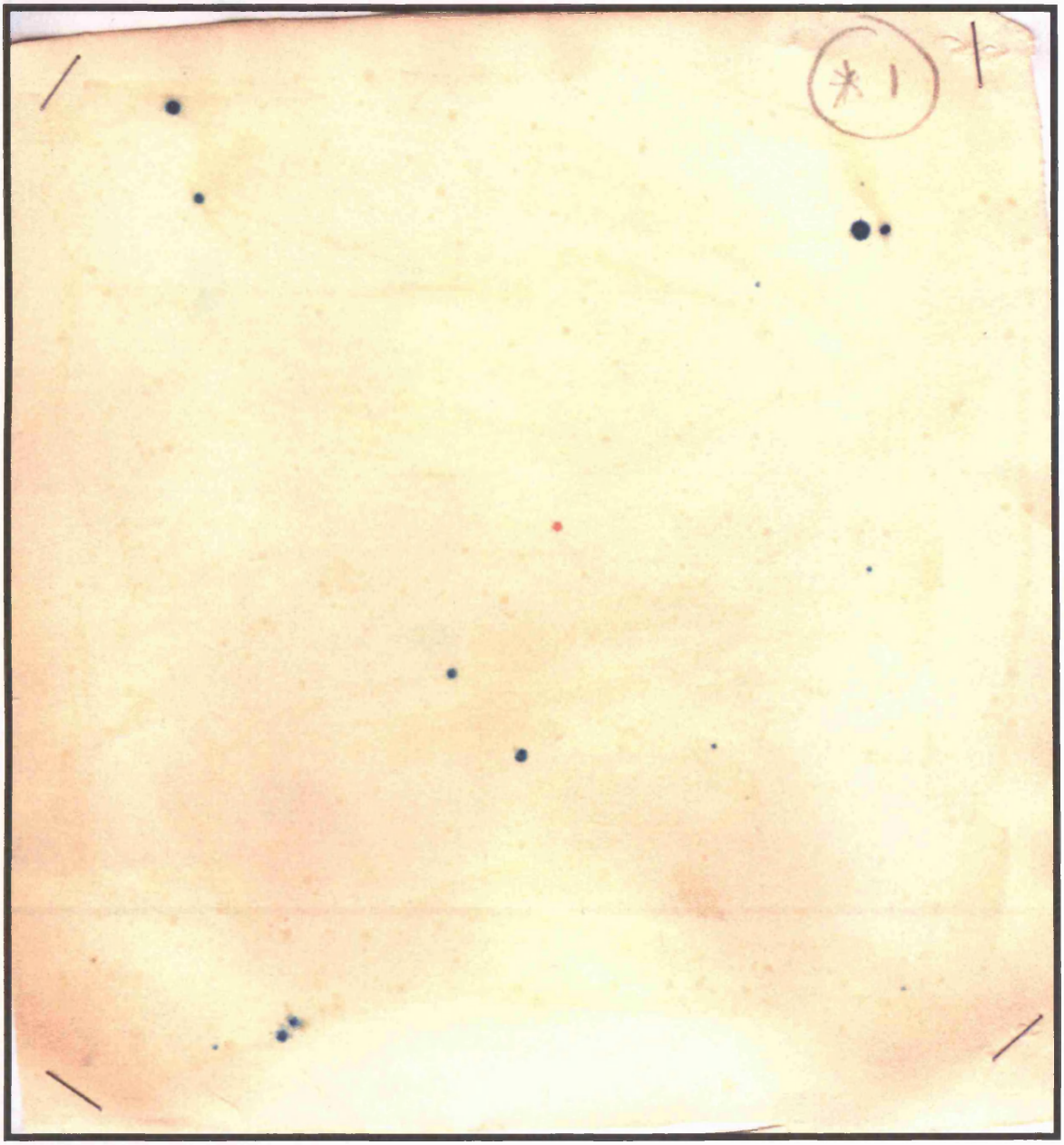


Plate 2

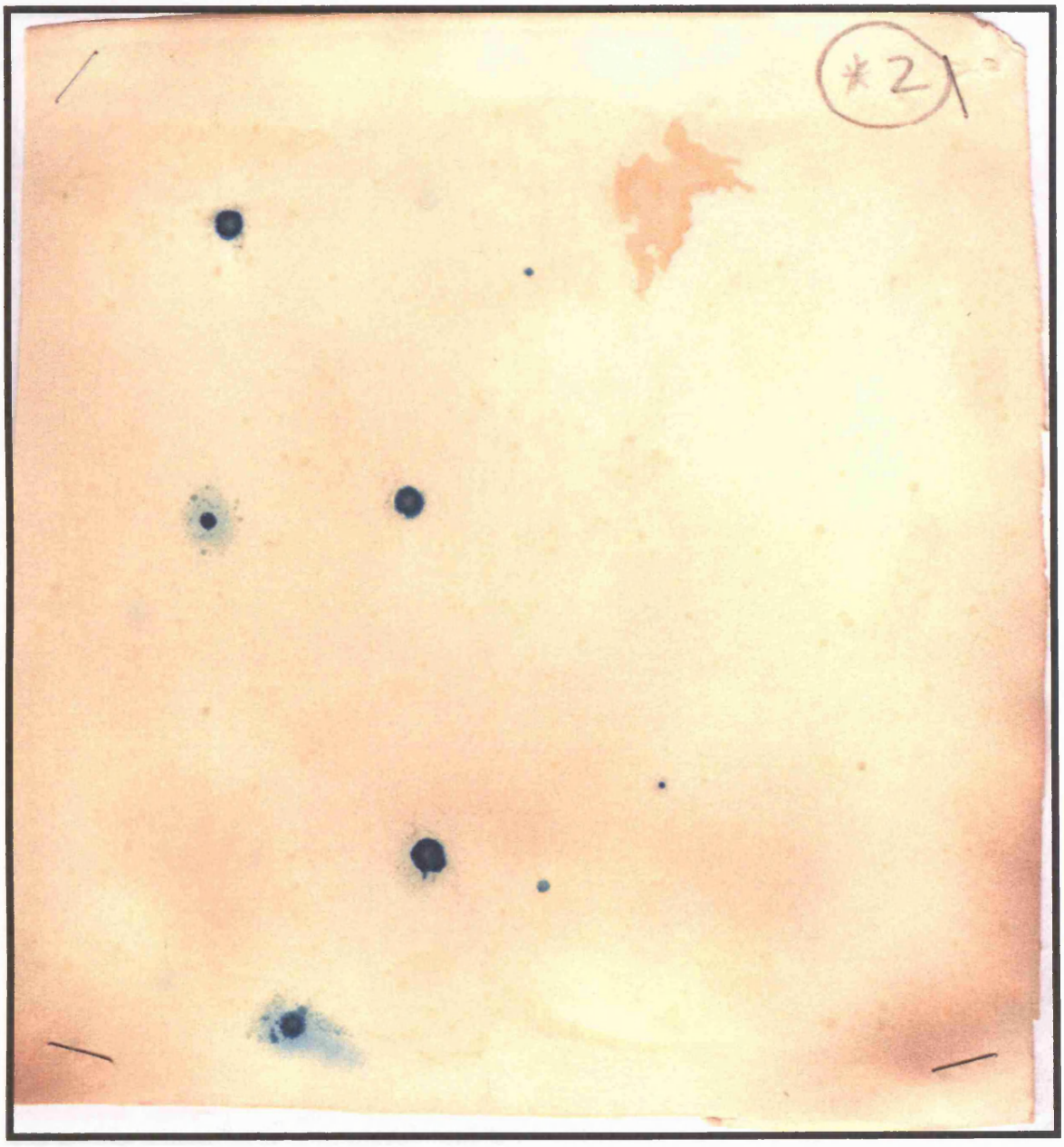


Plate 3

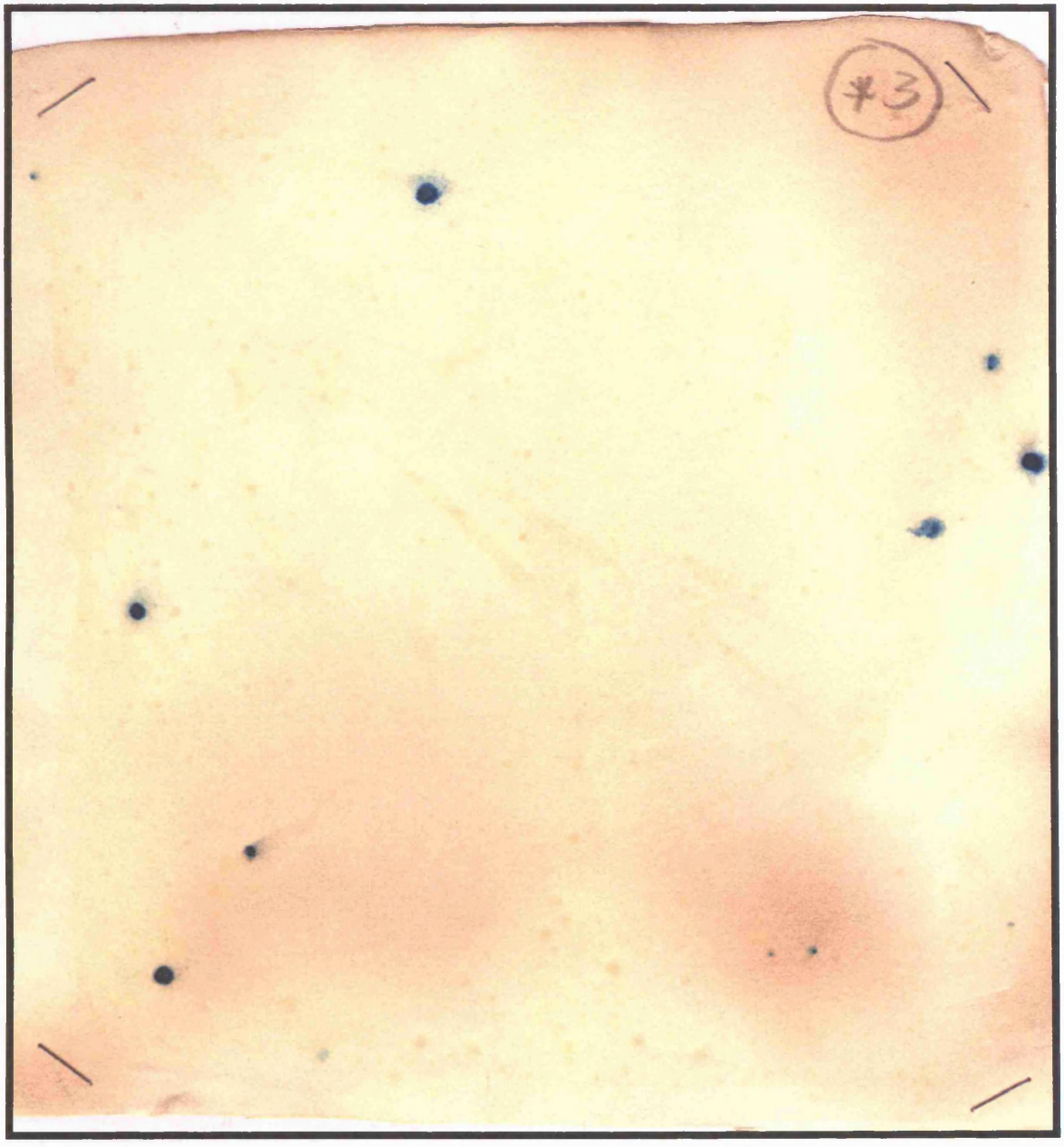


Plate 4

Plate 5

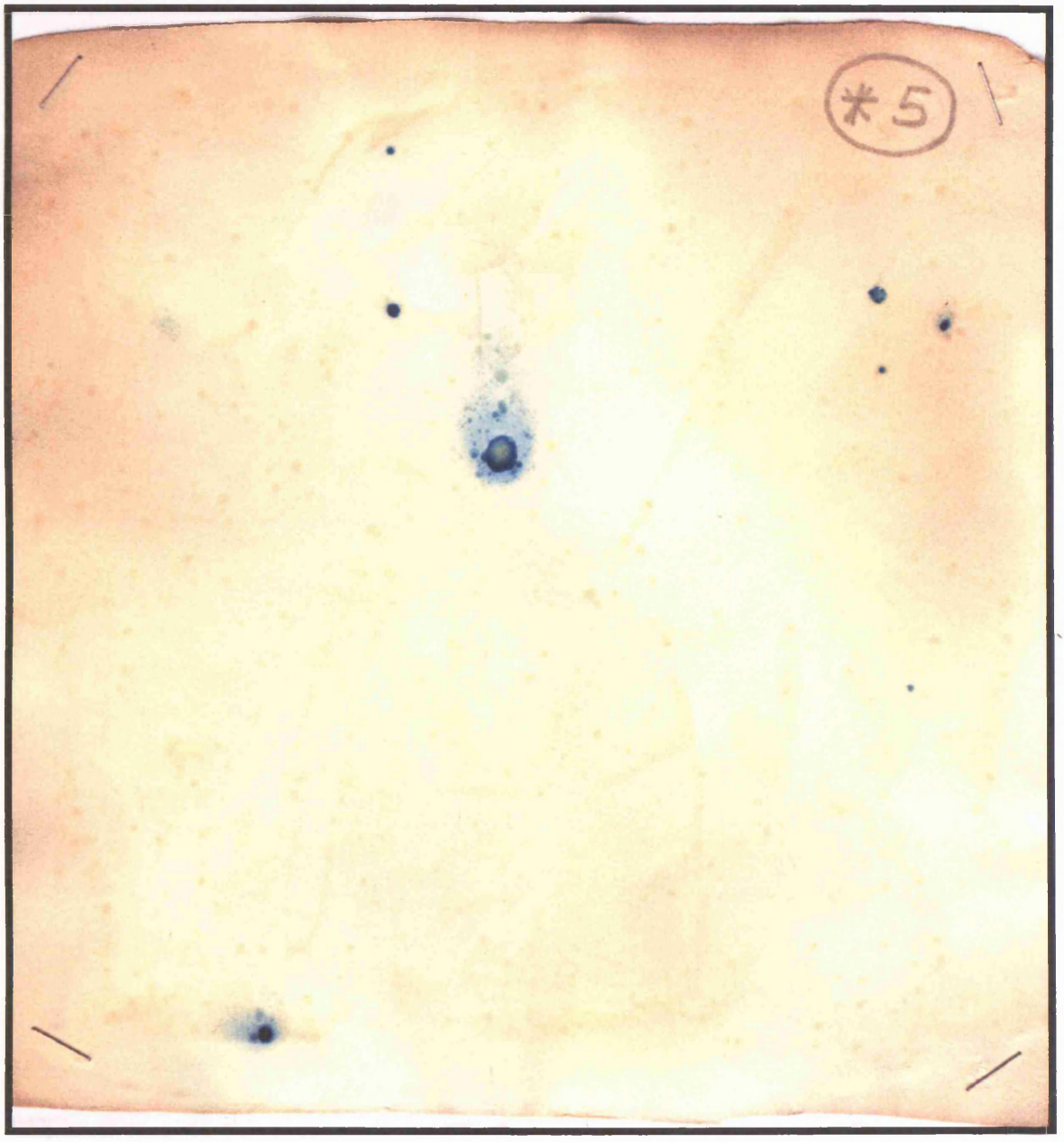


Plate 6

Plate 7

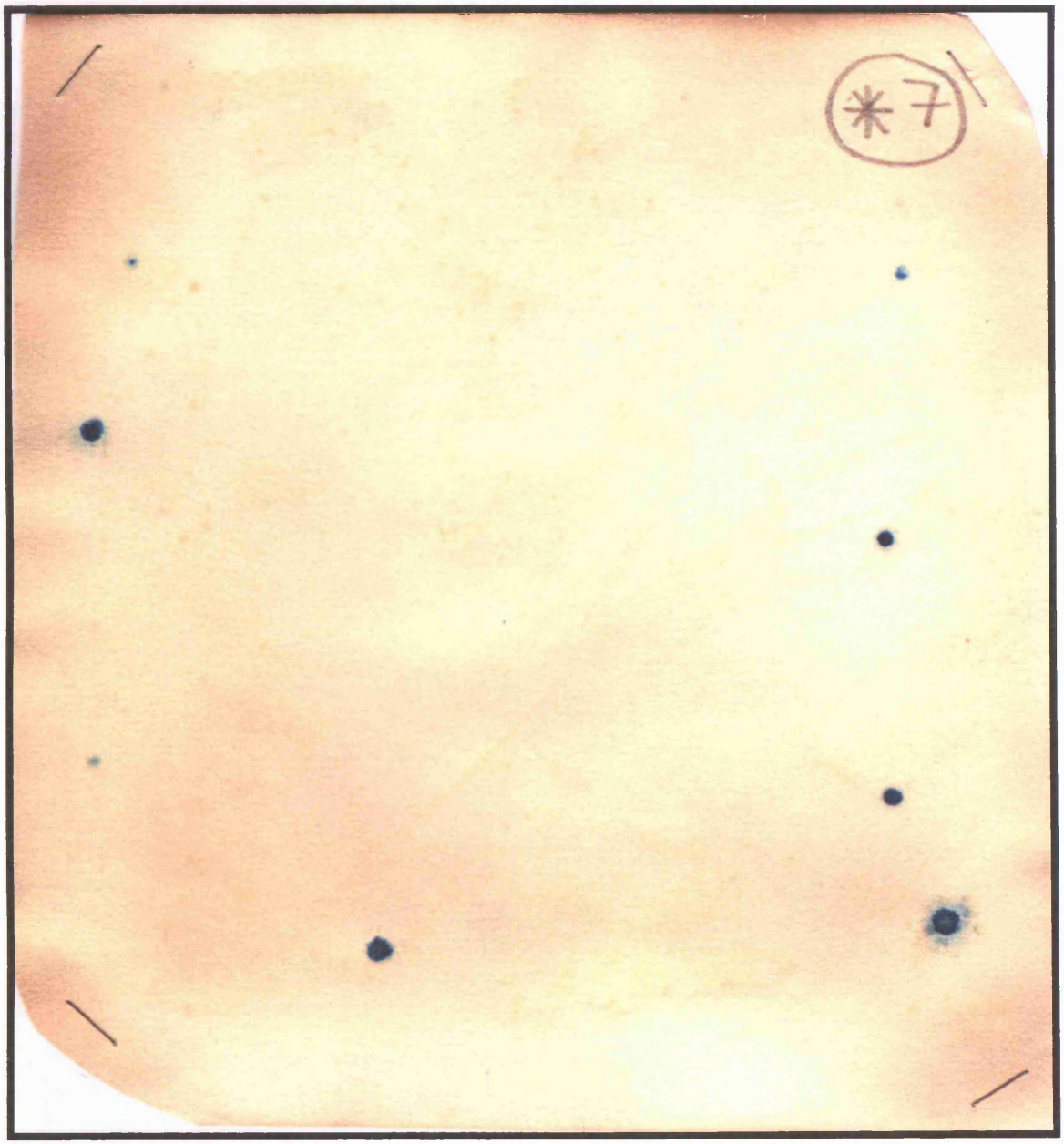


Plate 8

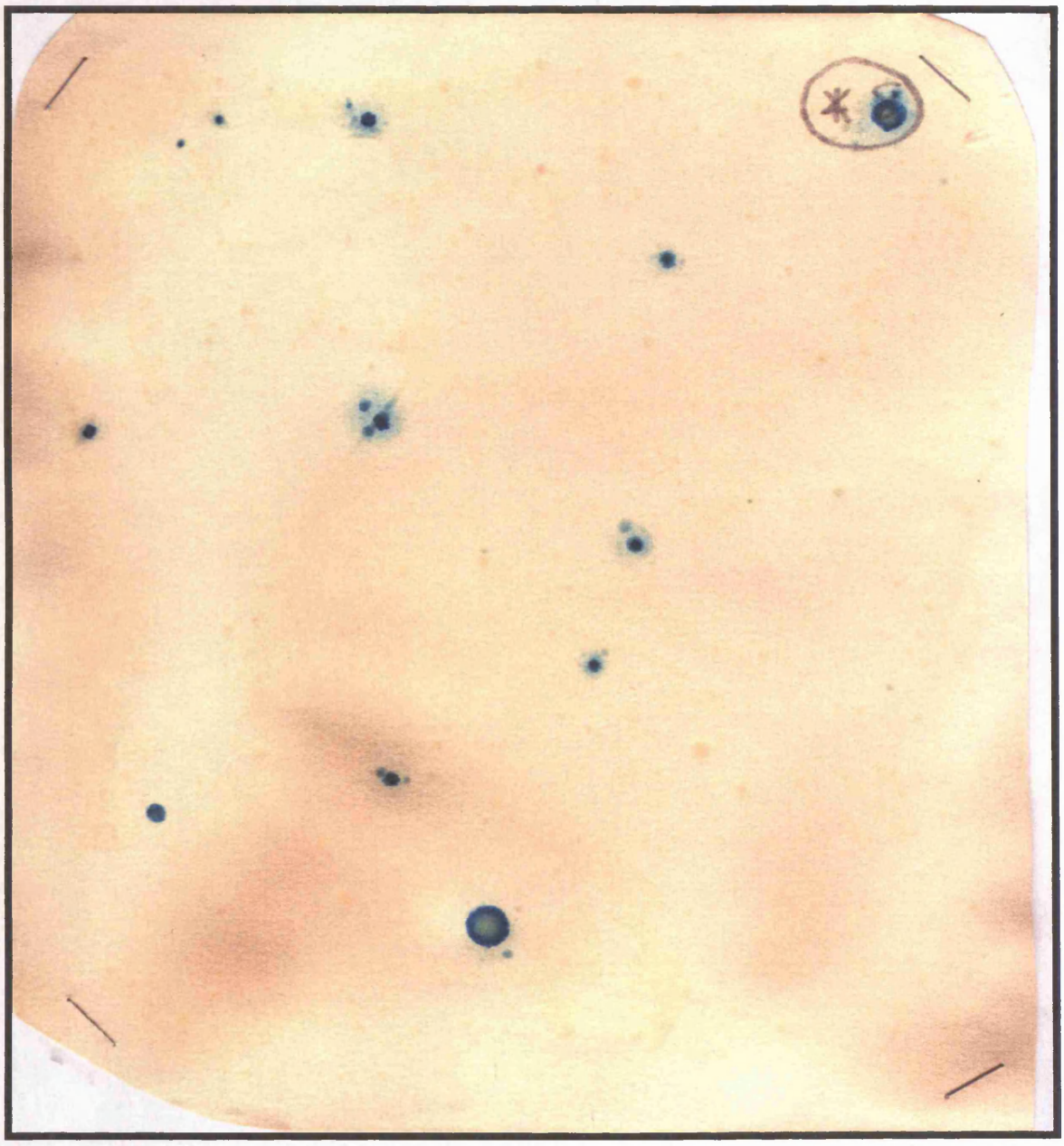


Plate 9

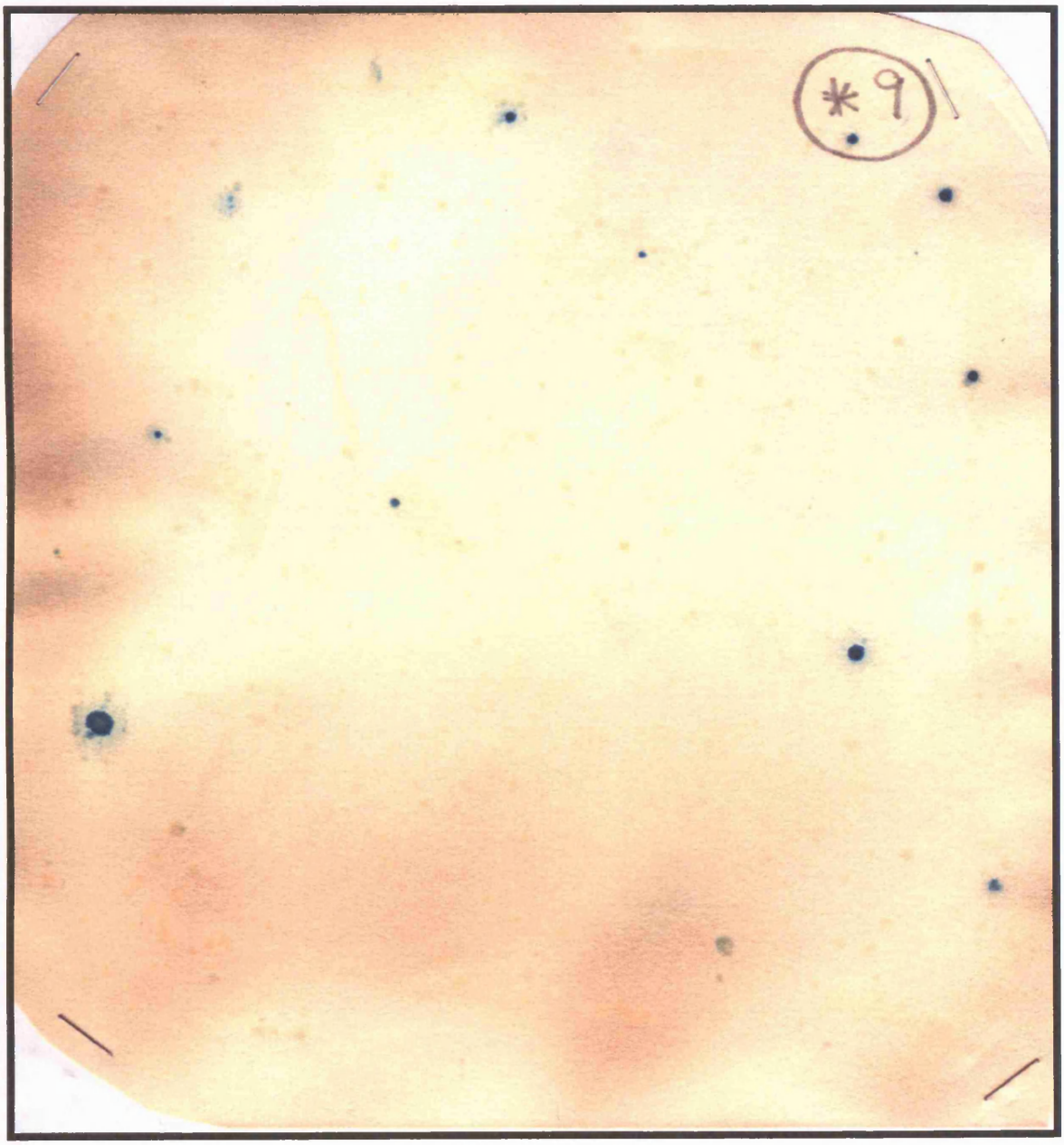
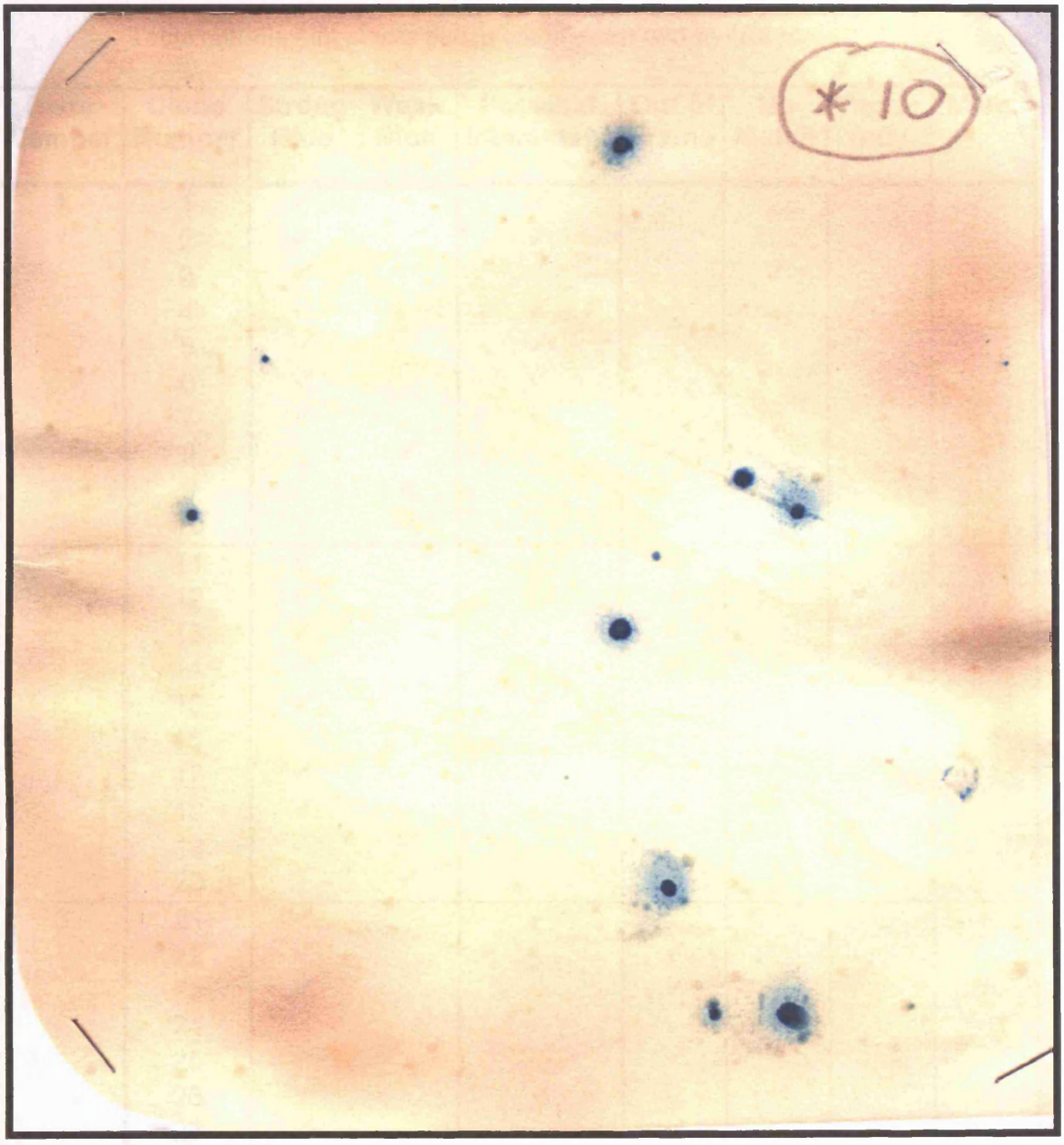


Plate 10



Appendix III

Table outlining all clones pulled out of yeast two-hybrid screen.

Plate Number	Clone Number	Strong Blue	Weak Blue	Possible Interactant	Out of Frame	No Match	Vector only	Misc.
1	1	✓		✓				
	2	✓		✓				
	3	✓				✓		
	4	✓		✓				
	5	✓		✓				
	6	✓		✓				
	7	✓		✓				
	8		✓		✓			
	9		✓		✓			
	10		✓		✓			
2	11	✓			✓			
	12	✓				✓		
	13	✓			✓			
	14	✓			✓			
	15	✓		✓				
	16	✓			✓			
	17	✓		✓				
	18	✓					✓	
	19		✓		✓			
	20		✓		✓			
3	21	✓		✓				
	22	✓			✓			
	23	✓			✓			
	24	✓				✓		
	25	✓			✓			
	26	✓		✓				
	27	✓			✓			
4	28	✓			✓			
	29	✓		✓				
	30	✓			✓			
	31	✓		✓				
	32		✓		✓			
	33		✓		✓			
5	34	✓					✓	
	35	✓			✓			

	36	✓			✓			
	37	✓		✓				
	38	✓					✓	
	39	✓			✓			
	40	✓			✓			
	41	✓			✓			
	42		✓		✓			
	43		✓		✓			
6	44	✓			✓			
	45	✓		✓				
	46	✓		✓				
	47	✓			✓			
	48	✓		✓				
	49		✓		✓			
	50		✓		✓			
	51		✓		✓			
	52		✓		✓			
	53		✓		✓			
	54		✓		✓			
	55		✓		✓			
7	56	✓		✓				
	57	✓			✓			
	58	✓			✓			
	59	✓		✓				
	60	✓			✓			
	61	✓			✓			
	62	✓		✓				
	63		✓		✓			
	64		✓			✓		
	65		✓	✓				
8	66	✓		✓				
	67	✓		✓				
	68	✓			✓			
	69	✓			✓			
	70	✓						✓
	71	✓			✓			
	72	✓			✓			
	73	✓			✓			
	74	✓		✓				
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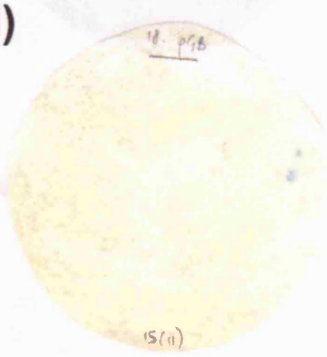
	76	✓			✓			
	77	✓			✓			
	78	✓						✓
	79		✓		✓			
	80		✓		✓			
	81		✓			✓		

	82		✓	✓				
	83		✓			✓		
	84		✓		✓			
9	85	✓		✓				
	86	✓						✓
	87	✓		✓				
	88	✓			✓			
	89	✓		✓				
	90	✓			✓			
	91	✓				✓		
	92	✓						✓
	93	✓					✓	
	94	✓			✓			
	95		✓				✓	
	96		✓		✓			
	97		✓	✓				
	98		✓	✓				
10	99	✓		✓				
	100	✓		✓				
	101	✓			✓			
	102	✓			✓			
	103	✓		✓				
	104	✓			✓			
	105	✓			✓			
	106	✓			✓			
	107	✓			✓			
	108		✓		✓			
	109		✓		✓			
	110		✓		✓			
	111		✓			✓		
	112		✓	✓				
	113		✓		✓			

Appendix IV

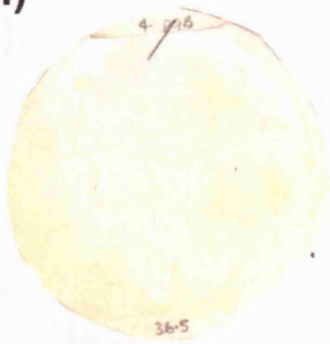
Summary of retest results for the yeast two-hybrid screen

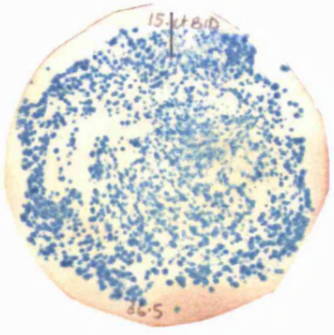
(i)



NDPK

(ii)

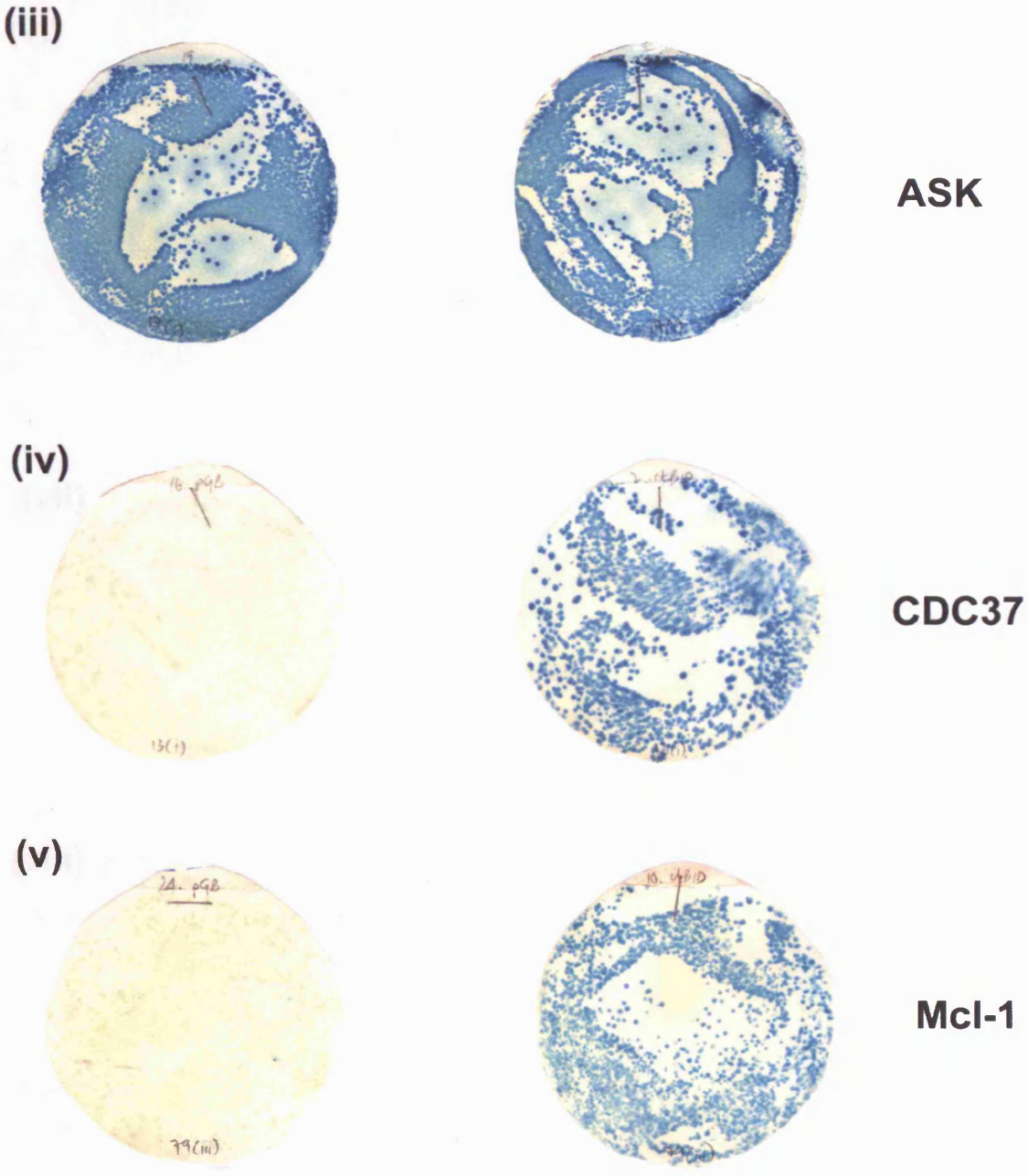




EID-1

- tBID

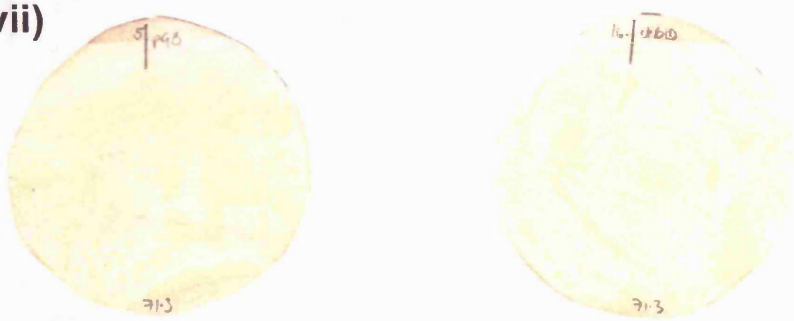
+tBID


(iii)
ASK
(iv)
CDC37
(v)
Mcl-1
- tBID
+tBID

(vi)

Bcl-2

(vii)



Thymic Factor

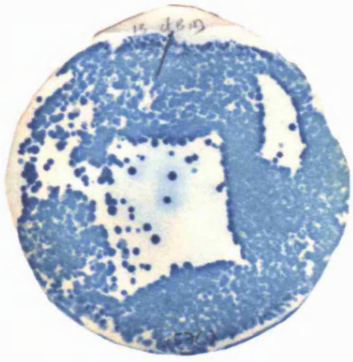
(viii)

Hypo. Protein

- tBID

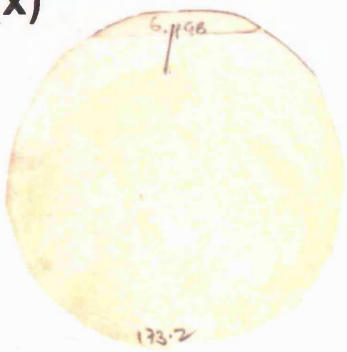
+tBID

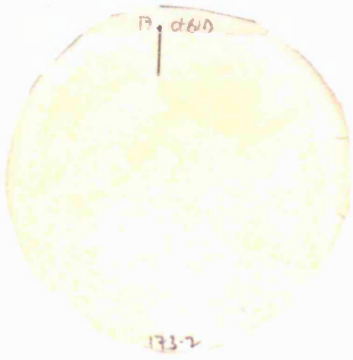
(ix)



CDC2L5

(x)





Rpl13a

- tBID

+tBID

bjh research paper

Characterisation of Mcl-1 cleavage during apoptosis of haematopoietic cells

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Summary

Mcl-1 is essential for normal haematopoiesis, being required for lymphocyte development and maintenance. Its role in haematopoietic differentiation and development is associated with its function as an anti-apoptotic member of the Bcl-2 family of proteins although the underlining mechanism is poorly understood. We have characterized caspase cleavage of the Mcl-1 protein during apoptosis. Caspase cleavage resulted in the removal of the PEST regions from the protein and generation of a fragment containing the BH-1, -2 and -3 homology domains. Removal of the PEST regions did not appear to alter Mcl-1 stability, suggesting that these regions are not responsible for Mcl-1's short half-life. In addition, unlike cleavage of Bcl-2 and Bcl-X_L, which resulted in pro-apoptotic fragments, cleaved forms of Mcl-1 were unable to induce apoptosis. This novel regulation of Mcl-1 may have important implications not only for its role in apoptosis but also for the essential role it plays in the differentiation and development of haematopoietic cells.

Keywords: apoptosis, Mcl-1, caspase, Bcl-2, haematopoiesis.

Mcl-1 was first identified as an early response gene switched on in the human myeloid leukaemia cell line ML-1, following treatment with the differentiation agent phorbol 12-myristate 13-acetate (PMA) (Kozopas *et al*, 1993). It has since established itself as an essential molecule not only early in development (Okita *et al*, 1998; Rinkenberger *et al*, 2000) but also for successful differentiation of haematopoietic cell types (Craig, 2002; Opferman *et al*, 2003). Mcl-1 has been shown to be absolutely required for the successful development and maintenance of B and T lymphocytes (Opferman *et al*, 2003). Conditional knock-out of *mcl-1* in B and T lymphocyte lineages results in severe loss of mature lymphocytes. In addition, knocking out the gene in mature lymphocytes shows that *mcl-1* is also essential for their survival in response to cytokine signalling. *mcl-1* transgenic mice are highly susceptible to developing B-cell lymphoma (Zhou *et al*, 2001) and there is a growing body of evidence to support Mcl-1 as an essential survival protein in multiple myeloma (MM) (Derenne *et al*, 2002; Zhang *et al*, 2002; Jourdan *et al*, 2003). Mcl-1 is regulated by a number of cytokines. Indeed interleukin (IL)-7, and to a lesser extent IL-15, induce strong expression of Mcl-1 in primary thymocytes that is important for their survival and maintenance (Opferman *et al*, 2003). Many MM cell lines are dependant on IL-6 for survival through upregulation of *mcl-1* expression. Downregulation of the Mcl-1 protein following treatment with antisense has been shown to induce apoptosis in these cells (Jourdan *et al*, 2003). Thus deregulation of Mcl-1 can have a profound effect on cell growth and survival.

As an anti-apoptotic member of the Bcl-2 family of proteins Mcl-1 also has a role in apoptosis and it is highly likely that its role in haematopoietic differentiation and development is linked to its function as an anti-apoptotic protein (Craig, 2002). The ability of Mcl-1 to protect cells from death is not very well understood. Like Bcl-2, it has all four Bcl-2 homology (BH) domains (Kozopas *et al*, 1993). It also has a C-terminal hydrophobic domain that is responsible for the localization of Mcl-1 to membranes within the cell, in particular to the mitochondrial membrane (Yang *et al*, 1996; Akgul *et al*, 2000). While it appears that Mcl-1 may not be as potent a protector against apoptosis as Bcl-2 (Reynolds *et al*, 1996), it does appear to be the main anti-apoptotic protein in some cell types including neutrophils (Moulding *et al*, 1998). Unlike other Bcl-2 family members, Mcl-1 contains a large region consisting of two PEST sequences that have been thought to target Mcl-1 for rapid degradation within the cell. Our results showed that in FDCP-1 cells Mcl-1 has a very rapid turnover, and that C-terminal fragments lacking these PEST regions are just as labile as the full length wild-type Mcl-1 in these cells. Although it is not clear exactly how Mcl-1 functions as an anti-apoptotic protein, recent data places Mcl-1 upstream of Bcl-X_L in response to ultraviolet (UV)-induced apoptosis of Hela cells (Nijhawan *et al*, 2003). Overexpression of Mcl-1 prevents translocation of Bax to the mitochondria, and while Bcl-X_L is unable to prevent this translocation it does inhibit oligomerization of translocated Bax in the mitochondria (Nijhawan *et al*, 2003).

The expression of Mcl-1 can be regulated at a transcriptional as well as a post-translational level. Its expression is increased in response to signalling from a number of different cytokines (Chao *et al*, 1998; Moulding *et al*, 1998; Huang *et al*, 2000; Puthier *et al*, 2001; Pelletier *et al*, 2002; Tumang *et al*, 2002). A recent study shows that Mcl-1 is rapidly degraded by the proteasome during induction of apoptosis by UV irradiation in Hela cells (Nijhawan *et al*, 2003). Here we report another novel regulation of Mcl-1 at a post-translational level, by caspase cleavage.

In most cases caspase activation is required for complete apoptosis of the cell (Budihardjo *et al*, 1999; Salvesen, 2002). Caspases exist as inactive pro-enzymes within the cell. Upon a death signal, large signalling complexes form and activate initiator caspases. These initiator caspases cleave a large number of substrates as well as activating downstream executioner caspases. A number of Bcl-2 family members are known to be cleaved by caspases. Bid, a BH-3-only protein, is activated upon cleavage by caspase-8 (Li *et al*, 1998; Luo *et al*, 1998), while Bcl-2, Bcl-X_L, BimEL and Bad have also been shown to be cleaved by caspases (Cheng *et al*, 1997; Clem *et al*, 1998; Condorelli *et al*, 2001; Chen & Zhou, 2004). Cleavage of Bcl-2 and Bcl-X_L result in the removal of their BH-4 domain to generate Bax-like fragments that contain BH-1, -2 and -3 homology domains. In all cases, cleavage of these proteins results in a stronger pro-apoptotic signal.

We found that cleavage of Mcl-1 by caspases did not result in an apoptotic signal. More importantly, caspase cleavage generated a fragment that, although structurally similar to Bax, did not display pro-apoptotic activity. In addition, we have found the PEST regions in Mcl-1 do not appear to be responsible for the short half-life in FDCP-1 cells. These important observations may lead to a better understanding of how the Mcl-1 protein functions in preventing apoptosis and facilitating its role in haematopoietic maintenance.

Materials and methods

Reagents

All media and cell culture reagents were obtained from Gibco (Glasgow, UK). All other reagents were purchased from Sigma (Poole, UK) unless otherwise stated.

Cell lines and culture

Jurkat T-cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mmol/l L-glutamine, 0·5 U/ml penicillin and 0·5 μg/ml streptomycin. FDCP-1 cells (American Type Culture Collection) were grown in RPMI 1640 medium supplemented with 10% FCS, 2 mmol/l L-glutamine, 0·5 U/ml penicillin, 0·5 μg/ml streptomycin, 1 mmol/l HEPES, 0·0004% β-mercaptoethanol and 10% IL-3 conditioned media from WEHI-3B cells. The 293-derived LinXE virus packaging cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 2 mmol/l L-glutamine, 0·5 U/ml penicillin, 0·5 μg/ml streptomycin and 75 μg/ml hygromycin (Calbiochem, San Diego, CA, USA). All cell lines were maintained at 37°C with 5% CO_2 unless otherwise stated.

Mcl-1 constructs and plasmids

The plasmid vector pcDNA3·1-HA was used for all *in vitro* assays and transient transfection experiments. This was generated by first annealing the two oligos 5′-TCGAG-ACCATGGCTTATCCTTATGACGTGCCTGACTATGCCAG-CCTGG-3′ and 5′-AATTCCAGGCTGGCATAGTCAGGCAC-GTCATAAGGATAAGCCATGGTC-3′. This double stranded oligo was then ligated into the *Xho* I and *Eco*RI sites of pcDNA3·1/*myc*-HIS©(-) B (Invitrogen, Paisley, UK). Full length human and mouse *mcl-1* cDNAs were cloned by polymerase chain reaction (PCR) in frame and downstream of the HA tag into the *Eco*RI and *Bam*HI sites of pcDNA3·1-HA. Both human and mouse *mcl-1* cDNAs were amplified by PCR using the primers 5′MCL-1- *Eco*RI (5′-CGGAATT-CATGTTGGCCTC-3′) and 3′MCL-1- *Bam*HI (5′-GCGGATCCCTTCTTATTAGATATGC-3′). Mutant *mcl-1* constructs D127A MCL-1 and D157A MCL-1 were generated by site directed mutagenesis using the GeneTailor™ Site-Directed Mutagenesis System from Invitrogen. The primer pairs used for D127A-MCL-1 were D127A-F (5′-CAT-GTCGCCCGAAGAGGAGCTGGCCGGGTACGAGC-3′) and D127A-R (5′-CAGCTCCTCTTAGGGCGACATGATGGCGT-CAGC-3′). The primer pairs used for D157A-MCL-1 were D157A-F (5′-GGTAATAACACCAGTACGGCCGGGTCAC-TACCC-3′) and D157A-R (5′-CGTACTGGTGTTAT TACCAGATTCCCCGACC-3′). A double mutant molecule (DblMut-MCL-1) was generated using the D127A-MCL-1 as a template and mutating Asp^{157} to Ala using the primers D157A-F and D157A-R as before. The caspase cleavage fragments of *mcl-1* were cloned by PCR into the *Eco*RI and *Bam*HI sites of pcDNA3·1-HA. 127-MCL-1 was amplified using the primer pair 5′Mcl-1- *Eco*RI and MCL-127-R (5′-CGCGGATCCCTA-GTCCAGCTCCTCTTCGG-3′), Δ127-MCL-1 was amplified using MCL-Δ127-F (5′-CCGGAATTCGGGTACGAGCCGGA-GCCTCTCGG-3′) and 3′Mcl-1- *Bam*HI, and Δ157-MCL-1 was amplified using MCL-Δ157-F (5′-CCGGAATTCGGGT-CACTACCCTCGACGC-3′) and 3′MCL-1-*Bam*HI. For retroviral infection of FDCP-1 cells a modified pMSCVneo vector, pMSCV-IRES-huCD2 tailless (a gift from Dr Owen Williams, Institute of Child Health) was used. This vector was generated by digesting the parental pMSCVneo with *Eco*RI and *Bam*HI

 doi:10.1111/j.1365-2141.2004.04949.x

and inserting a linker PCR fragment containing an internal ribosome entry site (IRES) followed by a human CD2 gene lacking most of its cytoplasmic tail. This enables the detection of infected cells by expression of human CD2 on the surface of infected cells. HA-Mcl-1, HA-127-Mcl-1, HA-Δ127-Mcl-1, HA-Δ157-Mcl-1 and HA-DblMut-Mcl-1 were all cloned by digesting the pcDNA3·1-HA constructs with *Xba*I, blunt ending, and then cloning into a blunt ended *Eco*RI site of pMSCV-IRES-huCD2.

Reverse transcription polymerase chain reaction (RT-PCR) analysis of mcl-1 constructs

The RNA was prepared from 3×10^6 cells for each of non-transduced FDCP-1 cells, or FDCP-1 cells transduced with pMSCV- HA-Mcl-1-IRES-huCD2, pMSCV-HA-Δ127-MCL-1-IRES-huCD2, or pMSCV-HA-Δ157-MCL-1-IRES-huCD2 using a Quiagen RNeasy mini kit. Reverse transcription was carried out using an oligo-dT primer and Superscript II reverse transcriptase (Invitrogen) according to the manufacturers instructions. The resulting cDNA was used for PCR to amplify β-Actin using the primer pair Actin F3 (5′-GGCATCCAC-GAAACTACCTTCAA-3′) and Actin B3 (5′-AACCGACTGC-TGTCACCTTCAC-3′); while a C-terminal region from over-expressed Mcl-1 constructs were amplified using the primer pair MCL-1-3F (5′-TGCAGCGCAACCACGAGAC-CGG-3′) and pcDNA3·1-R (5′-AAAGCTTGGTACCGAG-CTCGG-3′).

Western blot analysis

Protein samples were prepared by lysing cells in sample buffer. Sodium dodecyl sulphate (SDS)-polyacrylamide gels (10–15%) were prepared essentially as described (Laemmli, 1970). Proteins separated on the gels were transferred to either polyvinylidene difluoride (Immobilon-P; Millipore, Watford, UK) or nitrocellulose filters (Hybond-C pure; Amersham, Bucks, UK). Filters were washed and stained with anti-mcl-1 (S-19; Santa Cruz Biotechnology), anti-PARP (BD Pharmingen, Santa Cruz, CA, USA), anti-HA (clone 3F10; Roche, Lewes, UK), or anti-α-tubulin (clone YL1/2; Serotec, Oxford, UK). Horseradish peroxidase (HRP) conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. Filters were developed using an enhanced chemiluminescence (ECL) Western blotting detection reagent (Amersham).

Induction of apoptosis in Jurkat cells

For apoptosis assays cells were seeded at $1–2 \times 10^6$ cells/ml and treated with 300 ng/ml CH11 anti-Fas (Upstate Biotechnology, Milton Keynes, UK), 25 μmol/l etoposide or 1 μmol/l staurosporine for up to 6 h. After treatment, cells were harvested and washed with ice-cold phosphate-buffered saline (PBS). One-fifth of the cells were analysed using flow cytometry by staining with Annexin V-phycoerythrin (PE) (BD Pharmingen) and 7-amino-actinomycin D (7AAD) to measure apoptosis. The other four-fifths were pelleted and lysed in sample buffer for analysis by Western blot.

Preparation of apoptotic lysate and in vitro assay for Mcl-1 cleavage

Jurkat T-cells were seeded at 1×10^6 cells/ml and treated for 4 h with or without 300 ng/ml CH11 anti-Fas (Upstate Biotechnology). Cell lysates were prepared as described (Sun *et al*, 1999). Briefly, 2×10^7 cells were pelleted and washed in ice-cold PBS. Cells were then resuspended in PIPES (piperazine-1, 4-bis 12-ethanesulfonic acid) buffer [50 mmol/l PIPES/KOH (pH 6·5), 2 mmol/l ethylenediaminetetraacetic acid (EDTA), 0·1% (w/v) CHAPS and one tablet of complete inhibitors (Roche) per 10 ml lysis buffer] to a volume of 166 μl/10^8 cells. This was followed by three cycles of freezing in liquid nitrogen followed by thawing rapidly at 37°C. Samples were then spun in a benchtop centrifuge for 30 mins at 16 000 *g*. Supernatant was transferred to a fresh tube and spun at 100 000 *g* for 45 mins. The S100 fraction containing active caspases was collected and the protein concentration determined using the Bio-Rad protein assay. Aliquots were stored at −80°C. Human and mouse HA-Mcl-1 were *in vitro* translated using the TNT® T7 Quick Coupled Transcription/Translation System from Promega (Madison, WI, USA) in the presence of [^{35}S]-methionine (Amersham), according to the manufacturers instructions. Cell lysate was added to PIPES buffer to a final volume of 48 μl. For assays using recombinant active caspase-3 (BD Biosciences, Cowley, UK), active caspase-3 was added to a final volume of 48 μl with buffer used according to the manufacturers instructions. Caspase inhibitor zVAD-fmk (Enzyme System Products), or dimethyl sulphoxide as a control, was then added and incubated at 37°C for 15 min. Finally 1·0 μl of translated protein was added to the lysate, mixed by pipetting, and incubated at 37°C for 2 h. The reaction was terminated by the addition of 50 μl of 2 × sample buffer. Twenty microlitres of this was then resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Following this the gel was fixed, dried and exposed to a phosphoimager screen overnight.

Infection of FDCP-1 cells

Constructs for infection were first transfected into the LinXE packaging cell line using Lipofectamine (Invitrogen) according to the manafacturers instructions. Generally 8 μg of each construct was transfected into a 10 cm plate. Twenty-four hours after infection, the media on the cells was replaced by media used for the culture of FDCP-1 cells. Following incubation for an additional 24 h, 10 ml viral supernatant was collected for each construct. FDCP-1 cells for infection were counted and 4×10^4 cells resuspended in 2 ml viral supernatant containing 8·0 μg/ml polybrene to give a concentration of 2×10^4 cells/ml. Cells were spun at 360 *g* for

45 mins at 32°C and then incubated at 32°C with 5% CO_2 for 2 h. Most of the supernatant was carefully removed and a further 2 ml viral supernatant containing polybrene was added. The cells were spun as before and incubated at 32°C with 5% CO_2 for a further 4 h. The supernatant was again carefully removed and replaced with complete media and the cells incubated as normal overnight. The following morning media was removed from the cells and 4 ml viral supernatant containing polybrene was added and spun as before. Cells were incubated for 6 h at 32°C with 5% CO_2. Following this the media was replaced with complete media and the cells incubated as normal. The infected populations were expanded and infection efficiency was measured by flow cytometry using an anti-CD2-FITC antibody (eBioscience, San Diego, CA, USA). Generally, an infection efficiency of 80% upwards was observed.

Cycloheximide treatment of FDCP-1 cells and growth factor withdrawal assays

The FDCP-1 cells were split to 2×10^5 cells/ml the day before treatment. On the day of treatment 2 ml of cells at 5×10^5 cells/ml were placed in wells of a 6-well plate. Cells were then treated for 0, 10, 20, 30, 60, 90, or 120 min with 30 μg/ml cycloheximide (CHX), harvested and washed with ice-cold PBS before being lysed in Laemmli buffer. Samples were analysed by Western blots and probed with anti-Mcl-1 or anti-HA. Blots were also probed with anti-α-tubulin to check for loading. For growth factor withdrawal assays, FDCP-1 cells were prepared by first washing cells three times in RPMI-1640 medium. Cells were then resuspended in media containing RPMI-1640 with 1% FCS, 2 mmol/l L-glutamine, 0·5 U/ml penicillin, 0·5 μg/ml streptomycin, 1 mmol/l HEPES, and 0·0004% β-mercptoethanol and maintained at 37°C with 5% CO_2. Apoptosis was measured by harvesting cells, resuspending in hypotonic buffer and addition of propidium iodide to examine DNA content of the cells. The percentage of sub-G1 cells was used as a measure of apoptosis.

Results

Mcl-1 is cleaved during apoptosis in Jurkat cells

We initially observed that Mcl-1 was cleaved in Jurkat cells in response to treatment with the anti-Fas monoclonal antibody CH11. As shown in Fig 1A, Mcl-1 was cleaved from its full

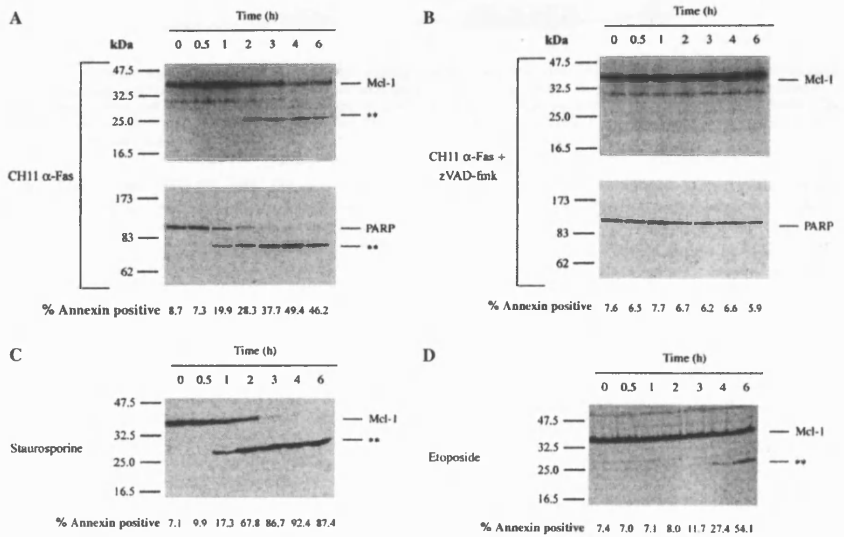

Fig 1. Mcl-1 is cleaved during apoptosis of Jurkat cells. Jurkat cells were treated with 300 ng/ml CH11 anti-Fas (A and B), 1·0 μmol/l staurosporine (C) or 25·0 μmol/l etoposide (D) over a 6-h time course. Apoptosis was quantified by flow cytometry using anti-Annexin V-PE. Total cell lysate was resolved by SDS-PAGE and Western blotted with an anti-Mcl-1 antibody (A, B, C and D) and an anti-PARP antibody (A and B). Pre-incubating with 75 μmol/l of the pan-caspase inhibitor zVAD-fmk completely abolished cleavage of Mcl-1 or PARP in response to CH11 (B). Asterisks indicate cleavage products.

length 42 kDa form to a lower band migrating at about 27 kDa as detected by Western blotting. A time course study showed that Mcl-1 cleavage occurred at the same time as PARP cleavage (Fig 1A). Inducing apoptosis with staurosporine (Fig 1C) or etoposide (Fig 1D) also resulted in the processing of Mcl-1. In order to establish if this cleavage was upstream or downstream of caspase activity, Jurkat cells were incubated with 75 μmol/l of the pan-caspase inhibitor zVAD-fmk for 30 min before inducing apoptosis. Both Mcl-1 and PARP cleavage were completely inhibited over the 6-h time course (Fig 1B). This indicated that caspase activity was required for Mcl-1 cleavage.

Mcl-1 cleavage is mediated by caspases

An *in vitro* system was set up to examine the processing of Mcl-1 more closely. Human (Fig 2A) or mouse (Fig 2B) *in vitro* translated [^{35}S]-HA-Mcl-1 was incubated with a cytoplasmic lysate from untreated Jurkat cells or Jurkat cells treated with CH11 anti-Fas antibody for 4 h. This lysate contained either inactive (i.e. non-apoptotic lysate) or active (i.e. apoptotic lysate) forms of caspases present in the cell. Treating the protein with apoptotic lysate clearly showed the cleavage of Mcl-1 (Fig 2A and B). Addition of zVAD-fmk to the apoptotic lysate completely inhibited this cleavage.

Human Mcl-1 was processed into three fragments while mouse Mcl-1 was processed into only two fragments. This suggests two cleavage sites in the human protein and only one in the mouse protein. Incubating human Mcl-1 with active caspase-3 resulted in the same cleavage pattern as for apoptotic lysate (Fig 2C), thus confirming that Mcl-1 could be directly cleaved by caspases and that the processing of Mcl-1 in Jurkat cells undergoing apoptosis was because of caspase cleavage of the protein. It is worth mentioning that although caspase-3 cleaved Mcl-1 *in vitro*, this may not be the primary or only caspase involved in Mcl-1 processing *in vivo*. A caspase cleavage consensus site (DXXD) was also present in the HA tag, which also appeared to be cleaved in our *in vitro* assays and could be easily seen by comparing the smallest fragment present on the gel (Fig 2C). Active caspase-3 appeared to cleave this site more efficiently than the apoptotic lysate and so this small fragment travelled slightly faster in this lane.

Analysis of the protein sequence of the human Mcl-1 protein did not identify any obvious or known caspase cleavage sites. All Asp residues were examined for a potential caspase cleavage site according to the three residues N-terminal to Asp and their homology to known caspase cleavage sites. A number of candidate cleavage sites were identified and the Asp residues were subsequently mutated by site directed mutagenesis to Ala residues. As shown in Fig 3A, mutation of D127A or D157A altered the cleavage pattern of the wild-type (wt) Mcl-1 protein when treated with apoptotic lysate (compare lanes 3 and 4 to lane 2). Generation of a double mutant (D127A, D157A Mcl-1) resulted in the complete abolition of Mcl-1 cleavage (Fig 3A, lane 5). The identical cleavage pattern was also observed by

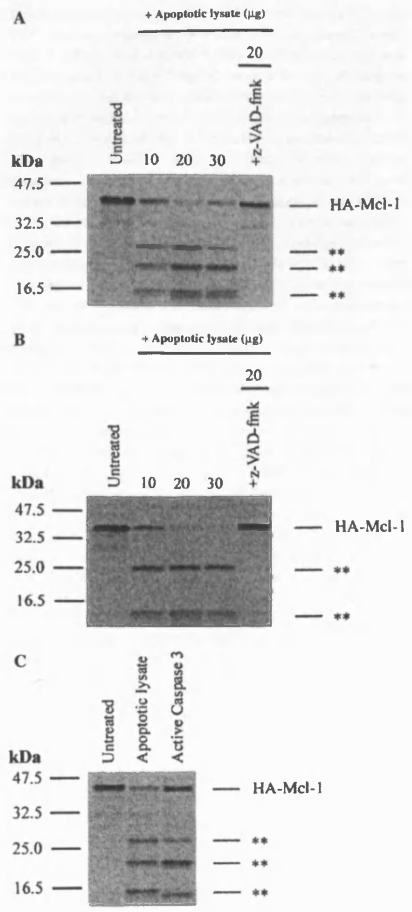

Fig 2. Human and Mouse Mcl-1 can be cleaved into a number of fragments by caspases. *In vitro* [^{35}S]-labelled human (A) and mouse (B) HA-Mcl-1 was incubated with different amounts of apoptotic lysate for 2 h at 37°C. Samples were analysed by resolving proteins with SDS-PAGE. The gel was dried and exposed to a phosphoimager screen. Pre-incubating with 75 μmol/l zVAD-fmk (final lane in both A and B) for 15 min prior to the addition of apoptotic lysate prevented this cleavage. (C) Treating human HA-Mcl-1 with an apoptotic lysate or with recombinant caspase-3 results in the same cleavage pattern. Asterisks indicate cleavage products.

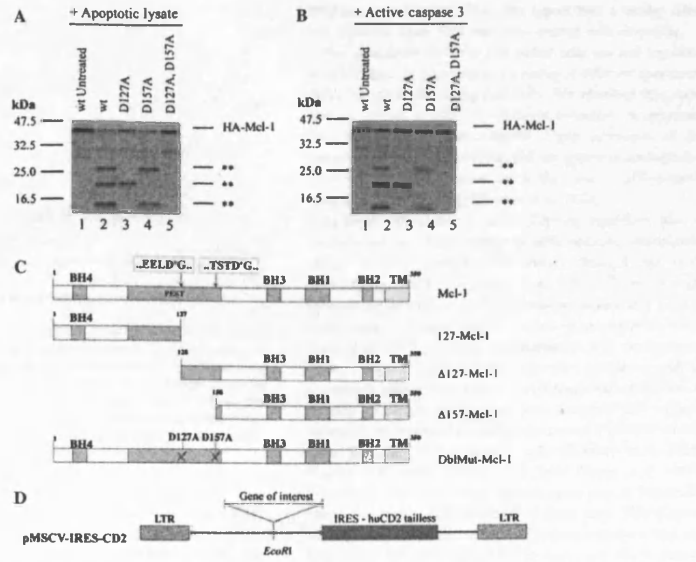

Fig 3. Human Mcl-1 is cleaved by caspases at D127 and D157. Aspartic acids D127 and D157 were mutated to alanine by site-directed mutagenesis. The mutant proteins were treated with apoptotic lysate (A) or active caspase-3 (B) as in Fig 2. This resulted in an alteration to the cleavage pattern by both apoptotic lysate and recombinant caspase-3. Cleavage was completely abolished when both residues were mutated (D127A, D157A). The fragments that were cloned for further analysis are depicted in (C). The pMSCV-IRES-huCD2 tailless vector used for retroviral infection is outlined in (D). Asterisks indicate cleavage products.

using active caspase-3 (Fig 3B). Thus the three fragments generated by cleavage of the human Mcl-1 molecule were (i) an N-terminal fragment from amino acids 1 to 127, (ii) a long C-terminal fragment from amino acids 128–350 (Δ127) and (iii) a shorter C-terminal fragment from amino acids 158–350 (Δ157) (Fig 3C). Western blots using the Santa Cruz anti-Mcl-1 (S-19) antibody clearly showed the Δ127 fragment (Fig 1) but not the shorter Δ157-fragment. This was because the epitope used for the generation of this antibody was a short sequence not present in the Δ157 fragment.

To examine further the functional consequences of Mcl-1 cleavage each of the cleavage products were cloned into the mammalian expression vector pcDNA3·1-HA. These constructs were used to transiently transfect Jurkat cells, which were then treated with CH11 or etoposide for up to 6 h. Apoptosis was measured by Annexin-V and 7AAD staining and analysed by flow cytometry. Mcl-1 did delay apoptosis, although it did not protect as well as Bcl-2 (data not shown). The C-terminal cleavage fragments tested also protected from apoptosis but did not show any significant difference in their ability to protect or promote apoptosis when compared with the wild-type Mcl-1 (data not shown). A caspase cleavage resistant mutant Mcl-1 was also tested in this system and gave very similar results to that of the wild-type Mcl-1 and the cleavage products.

Although we initially observed cleavage of Mcl-1 in Jurkat T-cells, its role in these cells is poorly characterized. Protection from apoptosis by Mcl-1 has been characterised in a number of cell types, including the murine IL-3 dependent haematopoetic cell line FDCP-1 (Reynolds *et al*, 1996; Zhou *et al*, 1997; Pedersen *et al*, 2002). We used these cells to further characterise the cleavage fragments and to examine the effect of the individual Mcl-1 cleavage products during growth factor withdrawal-induced apoptosis.

The PEST regions in Mcl-1 do not regulate its stability

Mcl-1 cleavage products were cloned into a retroviral pMSCV vector modified to contain a human CD2 gene lacking most of its cytoplasmic tail under the control of an IRES, thus enabling the detection of transduced cells by the presence of human CD2 on the cell surface (Fig 3D). FDCP-1 cells were transduced with constructs containing wild type and double mutant Mcl-1, Mcl-1 cleavage products and the empty vector

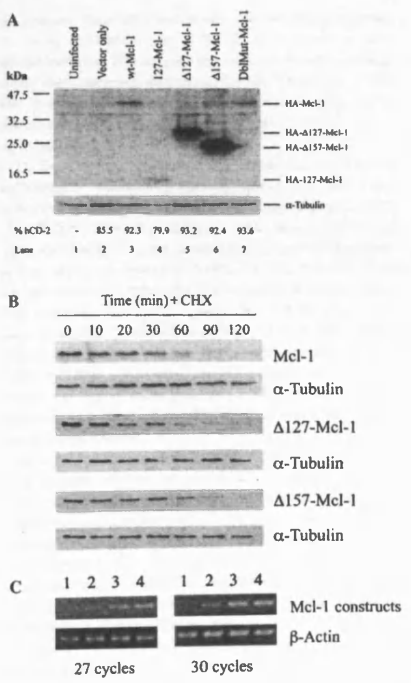

Fig 4. Mcl-1 stability is not regulated by its PEST regions in FDCP-1 cells. (A) FDCP-1 cells were infected with pMSCV-IRES-huCD2 constructs containing wild type HA-Mcl-1 (lane 3), HA-127-Mcl-1 (lane 4), HA-Δ127-Mcl-1 (lane 5), HA-Δ157-Mcl-1 (lane 6) and HA-DblMut-Mcl-1 (lane 7). Uninfected cells (lane 1) and vector only control cells (lane 2) were also used. Samples were prepared by lysing cell pellets in Laemmli buffer and resolving proteins on a SDS-PAGE gel. A Western blot was performed using an anti-HA antibody. The membrane was also probed with an anti-α-tubulin antibody to check for loading and the percentage of cells expressing the human CD-2 antigen was taken as a measure of infection efficiency. (B) FDCP-1 cells transduced with pMSCV-IRES-huCD2 vectors containing HA-Mcl-1 (top panels), HA-Δ127-Mcl-1 (centre panels) or HA-Δ157-Mcl-1 (bottom panels) were treated with 30 μg/ml cycloheximide (CHX) for the times indicated and samples analysed by Western blots using anti-Mcl-1 or anti-HA antibodies and an anti-α-tubulin antibody for loading. (C) RT-PCR reactions to assess the amount of RNA present in transduced cells. RT-PCR reactions on RNA from non-transduced FDCP-1 cells (lane 1), or FDCP-1 cells transduced with pMSCV-IRES-huCD2 vectors containing HA-Mcl-1 (lane 2), HA-Δ127-Mcl-1 (lane 3) or HA-Δ157-Mcl-1 (lane 4) after either 27 or 30 cycles of amplification.

as a control (Fig 4A). Infection efficiencies of 80–90% were routinely achieved and overexpressed protein was detected by Western blot using an anti-HA antibody (Fig 4A). We found that fragments lacking part (i.e. Δ127) or all (i.e. Δ157) of the PEST region appeared to be much more abundant (Fig 4A, lanes 5 and 6) when compared with full length wild-type and DblMut-Mcl-1 proteins (Fig 4A, lanes 3 and 7). Initially we thought that this was as a result of protein stability, especially as the PEST regions in Mcl-1 are thought to be responsible for the rapid turnover of Mcl-1. To further assess the stability of full length Mcl-1 and the Δ127-Mcl-1 and Δ157-Mcl-1 cleavage fragments, the mixed populations of transduced cells were treated with the translation inhibitor cycloheximide (CHX) for 0, 10, 20, 30, 60, 90 or 120 min (Fig 4B). The Western blots (Fig 4B) showed that Mcl-1 and the two cleavage fragments were surprisingly unstable, with a half-life of <30 min, while the levels of α-tubulin remained constant over the time course. The rate of degradation of each fragment was calculated for each construct using a (Bio-Rad, Hercules, CA, USA) GS-800 calibrated densitometer and Quantity One quantification software (data not shown). This analysis suggested that Δ157-Mcl-1 might be slightly more stable although this difference was not statistically significant, and suggested that the PEST region was not responsible for the rapid turnover of Mcl-1.

As protein stability could not account for the difference in protein levels in the transduced FDCP-1 cells we next examined levels of mRNA for each of the constructs. Figure 4C shows RT-PCR reactions to amplify a common C-terminal region present in the overexpressed wild-type Mcl-1 and all of cleavage fragments. RNA was prepared from four samples – non-transduced FDCP-1 cells (Fig 4C, lane 1) and FDCP-1 cells transduced with HA-Mcl-1 (Fig 4C, lane 2), HA-Δ127-Mcl-1 (Fig 4C, lane 3), or HA-Δ157-Mcl-1 (Fig 4C, lane4). The overall level of mRNA for full length Mcl-1 proved to be dramatically less than that for either Δ127-Mcl-1 or Δ157-Mcl-1 (Fig 4C, compare lane 2 with lanes 3 and 4). Amplification of β-actin by PCR was used to show that equal amounts of cDNA were used for PCR. As expected, non-transduced cells did not give any PCR product. Thus the differences in the amount of mRNA present in the cells were repeated in the protein levels (Fig 4A).

Products of caspase cleavage are not apoptotic

As the caspase cleavage of all other Bcl-2 family members known to undergo this process results in a pro-apoptotic signal, we thought that the generation of an Mcl-1 Bax-like fragment by caspase cleavage, containing only BH-1, -2 and -3 homology domains, may also give a pro-apoptotic phenotype to the Mcl-1 cleavage products. We carried out apoptosis assays using the transduced FDCP-1 cells to investigate if this was the case. Apoptosis was induced following growth factor withdrawal for a period of 5 d and monitored each day by measuring the percentage of sub-G1 cells using a flow cytometer. Contrary to previously published data (Zhou *et al*, 1997), Mcl-1 did not appear to delay apoptosis in these cells when compared with empty vector infected cells. Interestingly the fragments did not appear to have any pro-apoptotic

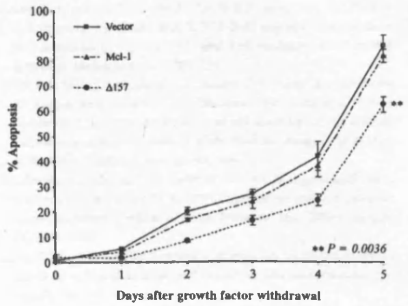

Fig 5. Caspase cleavage products do not induce apoptosis in FDCP-1 cells. Infected FDCP-1 populations were starved of growth factors over a period of 5 d. Apoptosis was measured by propidium iodide staining and flow cytometery. Results are expressed as percentages (mean of three independent experiments ± standard deviation). Of all the constructs infected, only HA-Δ157-Mcl-1 showed a significant difference to vector only control infections or wild type HA-Mcl-1 infections.

function. In fact, the Δ157 fragment appeared to show a slight protection from growth factor withdrawal-induced apoptosis when compared with empty vector control infected cells ($P = 0{\cdot}0036$ at day 5) (Fig 5). This suggestion, that the cleavage products may also be able to offer some protection from apoptosis, is a unique and novel finding for Mcl-1.

Discussion

Various transgenic models involving Bcl-2 family members have shown that these proteins have important roles in growth, development and haematopoiesis. This role is linked with the ability of these proteins to influence apoptosis, although exactly how they function to regulate apoptosis is not completely understood. For example the pro-apoptotic proteins Bax and Bak are essential for release of mitochondrial proteins required for apoptosis (Wei *et al*, 2001). Upon activation, these proteins translocate and concentrate in the outer mitochondrial membrane and homo-oligomerize to form larger molecular weight complexes (Antonsson *et al*, 2000; Eskes *et al*, 2000; Wei *et al*, 2000; Antonsson *et al*, 2001) resulting in the release of mitochondrial proteins (Gross *et al*, 1999). On the contrary, Bcl-2 is able to inhibit release of these mitochondrial proteins. Whether it does this by sequestering BH3-only proteins, preventing them from activating Bax and Bak or by inhibiting formation of the activated Bax/Bak complexes is still unclear (Cheng *et al*, 2001). A recent study has shown that Mcl-1 appears to function upstream of Bcl-X_L in response to UV-induced apoptosis, and complete disappearance of Mcl-1 is required before translocation of Bax to the mitochondria and activation of pro-apoptotic proteins (Nijhawan *et al*, 2003). They also report that a similar effect was observed when Hela cells were treated with etoposide.

Our data show that Mcl-1 in Jurkat cells was not regulated in this manner in response to a number of different apoptosis-inducing agents, including etoposide. We observed that there was no change in Mcl-1 levels upon induction of apoptosis until the caspases were activated. Upon activation of the caspases, Mcl-1 was cleaved but did not appear to be degraded prior to caspase activation as is the case in UV-induced apoptosis in Hela cells (Nijhawan *et al*, 2003).

As previously outlined, Mcl-1 plays an important role in development and commitment to differentiation particularly within the haematopoietic cell lineages (Rinkenberger *et al*, 2000; Craig, 2002; Opferman *et al*, 2003). It is strongly upregulated in response to differentiation signals and protein levels rapidly increase within the cell (Kozopas *et al*, 1993; Yang *et al*, 1996). Cellular differentiation and development require major changes in gene expression profiles as well as substantial changes to cellular morphology and structure. A number of recent publications have identified that caspase activity is required for the differentiation of a number of cell types including haematopoetic cells (Eckhart *et al*, 2000; Pandey *et al*, 2000; Zermati *et al*, 2001; Arama *et al*, 2003). These data show that activation of caspases play an important role in successful differentiation of these cells. This caspase activity shows selective cleavage of known substrates that are involved in cellular organization. In such cases where caspase activity is required for successful differentiation it is possible that Mcl-1 cleavage plays a role in maintaining cell viability, allowing reorganization and commitment to differentiation to proceed and preventing the cell from premature and inappropriate apoptosis.

Human Mcl-1 is cleaved at two sites *in vitro*, Asp^{127} and Asp^{157}. Mouse Mcl-1 is only cleaved at one site and this is likely to be at Asp^{108}, as the region before this residue appears to be conserved with the caspase cleavage site at Asp^{127} in human Mcl-1. We have shown that this cleavage can be mediated by recombinant caspase-3 or by apoptotic cell lysate although we have as yet been unable to detect the shorter Δ157-Mcl-1 fragment in intact cells undergoing apoptosis by Western blot. The Santa Cruz anti-Mcl-1 (S-19) antibody was raised against a peptide from amino acid 121–139 in human Mcl-1 and this only enables the detection of the larger Δ127-Mcl-1 fragment. This epitope is lost in the Δ157-Mcl-1 fragment and therefore the antibody was unable to detect this fragment.

Cleavage of Mcl-1 by caspases results in the generation of fragments that lack the PEST regions at the N-terminus of the protein. PEST domains are associated with targeting proteins for rapid degredation by the proteasome (Rechsteiner & Rogers, 1996) and Mcl-1's PEST domains are thought to be responsible for its short half-life (Kozopas *et al*, 1993; Yang *et al*, 1996). Our results show that the half-life of Mcl-1 is surprisingly short in FDCP-1 cells. Interestingly, removing the PEST regions from the protein, as was the case with the caspase cleavage fragments, did not significantly alter the turnover of

the protein. Thus full-length Mcl-1 and the caspase cleavage fragments Δ127-Mcl-1 and Δ157-Mcl-1 all appear to have similar half-lives. This suggests that some other region existing within the C-terminus determines the half-life of Mcl-1. This also raises questions as to how the PEST-containing region contributes to Mcl-1 function if it is not responsible for its rapid turnover.

The Bcl-2 family members Bid, Bad, BimEL, Bcl-2 and Bcl-X_L are known to be cleaved by caspases (Cheng *et al*, 1997; Clem *et al*, 1998; Li *et al*, 1998; Luo *et al*, 1998; Condorelli *et al*, 2001; Chen & Zhou, 2004). This cleavage is best characterized for Bid and is essential for its role as a pro-apoptotic protein. However, the role of caspase cleavage of BimEL, Bad, Bcl-2 and Bcl-X_L is less well understood although caspase cleavage of these proteins does result in a stronger apoptotic effect (Cheng *et al*, 1997; Clem *et al*, 1998; Condorelli *et al*, 2001; Chen & Zhou, 2004). This change in apoptotic potential of these proteins can only result in further committing the cell to apoptosis. Although caspase cleavage of Mcl-1 generates a Bax-like fragment (i.e. containing only BH-1, -2 and -3) it does not appear to have any pro-apoptotic activity.

As Mcl-1 has already been reported to protect FDCP-1 cells from apoptosis induced by growth factor withdrawal (Zhou *et al*, 1997) we overexpressed wild type or mutated Mcl-1 as well as the cleavage fragments in these cells. In contrast to what has previously been reported, we found no significant difference between control infected cells and those infected with a wild-type Mcl-1 construct. We repeated this experiment a number of times and consistently found this to be the case. It is possible that this is partly because of the fact that we have used a mixed population of infected cells rather than generating and selecting for high expressing clones that may be more resistant to apoptosis.

It was surprising that the caspase cleavage products showed no apoptotic effect, given that all other Bcl-2 family members that are known to be cleaved by caspases generate a stronger apoptotic response. In fact, the smaller Δ157-Mcl-1 C-terminal fragment appeared to protect FDCP-1 cells from apoptosis. After 5 d approximately 55–60% of the Δ157-Mcl-1 infected cells were apoptotic while apoptosis within the other populations of infected cells was much higher at 85–90%. This unique finding suggests that Mcl-1, unlike other Bcl-2 family proteins, may have a role to play in continuing to protect the cell even after caspase activation. Western blotting analysis of infected cells showed that both HA-Δ127-Mcl-1 and HA-Δ157-Mcl-1 were in much greater abundance than full-length HA-Mcl-1 molecules, although the levels of infection were similar. This appeared to be a result of higher mRNA levels in cells transduced with Δ127-Mcl-1 and Δ157-Mcl-1, which ultimately results in more protein expressed. The reason that Δ157-Mcl-1 has a stronger protective effect on the cells is probably partly because of the fact that there is substantially more protein present in the cell. However, if this were the only reason then the larger Δ127-Mcl-1 fragment should also protect to a greater extent than wild-type Mcl-1, as it is also in much greater abundance. We were unable to detect any significant increase in protection by the Δ127-Mcl-1 fragment and so an increased level of the protein may only be part of the reason for Δ157-Mcl-1's ability to delay apoptosis.

Our results show that the PEST regions in Mcl-1 do not affect its stability in FDCP-1 cells and that caspase cleavage of Mcl-1 results in the removal of these regions and generates a Bax-like fragment that can still protect cells from apoptosis. However the function of these caspase cleaved fragments is unclear. Further investigations will address how these cleavage fragments influence apoptosis and also determine the importance of these fragments in haematopoietic differentiation and development.

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